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14. ABSTRACT The project proposes a novel integrative preclinical approach to study risk factors for and neurobiology of post- traumatic stress and depression. Why risk factors? Because PTSD is the only psychiatric disorder to which there is seemingly a clear etiological agent - a trauma event that triggers it, most models of concentrate on what would constitute a trauma in the studied animals. However, because the majority of people exposed to traumatic experiences actually do not develop PTSD the exposure to the traumatic experience is necessary, but not a sufficient condition to induce the disorder. We focus on both distal (Childhood adversities) and proximal (Sleep restriction) potential risk factors, with high relevance to soldiers. The primary aims of the project are thus: 1) To establish an effective animal model of PTSD that would take into consideration risk factors to the induction of trauma 2) To examine the role of sleep restriction as an immediate risk factor in PTSD 3) To establish the role of childhood adversity as a long-term risk factor in PTSD, particularly in association with sleep restriction 4) To develop the model as a platform for pharmacological testing of novel targets for drug development 5) As an additional aim – once an effective animal model is established - to use in order to identify novel targets for drug development					
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Title: **Early life stress and sleep restriction as risk factors in PTSD –
An integrative preclinical approach**

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Introduction

The project proposes a novel integrative preclinical approach to study risk factors for and neurobiology of post-traumatic stress and depression.

Why risk factors? - Because PTSD is the only psychiatric disorder to which there is seemingly a clear etiological agent – a traumatic event that triggers it, most models of PTSD concentrate on what would constitute a trauma in the studied animals. However, because the majority of people exposed to traumatic experiences actually do not develop PTSD the exposure to the traumatic experience is necessary, but not a sufficient condition to induce the disorder. We focus on both distal (Childhood adversities) and proximal (Sleep restriction) potential risk factors, with high relevance to soldiers. The primary aims of the project are thus:

- 1) To establish an effective animal model of PTSD that would take into consideration the contribution of risk factors to the induction of the trauma.
- 2) To examine the role of sleep restriction as an immediate risk factor in PTSD.
- 3) To establish the role of childhood adversity as a long-term risk factor in PTSD, particularly in association with sleep restriction.
- 4) To develop the model as a platform for pharmacological testing of novel targets for drug development.
- 5) As an additional aim – once an effective animal model is established – to use it in order to identify novel targets for drug development.

Body

Task 4+6: The impact of sleep restriction on the outcome of exposure to UWT-

To test for the effects of sleep restriction we have employed we have adopted the well-established protocol of Meerlo et al, (2008). A thorough investigation could not identify any significant effects of sleep restriction on the long-term outcome of exposure to a traumatic event. We have run several additional experiments to make sure that the result obtained here is indeed the outcome (or lack of it) of the manipulation. Thus, contrary to the initial hypothesis, proximal sleep restriction as modeled in the current project is not a risk factor for later developing PTSD symptoms. We are in the process of summarizing this result, as we believe this is an important indication by itself.

The effect of sleep restriction on the short term behavioral response to a mild stressor

Following our results so far, indicating no substantial effects of sleep restriction (SR) on the long term reaction to underwater trauma (UWT), we speculated that UWT may be such a severe stressor, that SR cannot exacerbate its effects. Hence we decided to test the effect of SR on the short term reaction to a milder stressor – forced swim (FS). Our aim was to examine whether SR will exacerbate the short term reaction to FS.

Methods

Animals

24 Male Sprague Dawley rats (~8 weeks old, 250-275g) were used for the experiment. Animals were group housed at $22 \pm 2^\circ\text{C}$ under 12-h light/dark cycles. Water and food were available ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

Experimental groups

All rats were randomly assigned to one of the following experimental conditions:

1. SR prior to FS (SR+FS) – Rats exposed to SR, followed by FS.
2. SR Control prior to FS (FS) – Rats exposed to the control procedure of SR, followed by FS.
3. SR Control with no stressor (control) – Rats exposed only to the control procedure of SR.

Experimental design

Following delivery and acclimation to the vivarium, all rats were habituated to the SR apparatus by placing them on the wheels for 1 h on 3 consecutive days (slowly or voluntary rotating wheels, according to the experimental group). Following wheels habituation, group SR+FS was exposed to SR for 8 days and the other groups were exposed to the control procedure of SR. After 8 days of SR, rats from the relevant groups were exposed to FS. 3 days later, rats were assessed for anxiety level using open field, elevated plus maze and acoustic startle testing. Timeline of procedures is presented in figure 1.

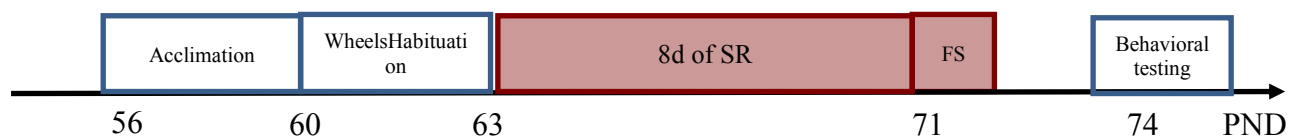


Figure 1: Timeline of procedures.

Procedures and assessments

Sleep restriction

In this protocol (adapted from Meerlo et al., 2008) SR was performed by confining the rats in slowly rotating wheels (diameter 35.5 cm, approximately 1.5 meter per minute, model 80860A; Lafayette Instruments Company, Lafayette, IN, USA). Control rats were placed in voluntary rotating wheels (model 80860W). The rats had continuous access to food and water at the side of the wheel. Rats were placed on the wheels for 20

hrs a day for 8 days. They were allowed to sleep in their home cages between 10:00-14:00, the first 4 hrs of the light phase. The remaining 20 hrs of the day, they were on the wheels.

Forced swim stressor

15 min forced swim in a plastic tank (diameter 40 cm, height 45 cm) containing 40 cm of water at $22\pm 2^{\circ}\text{C}$.

The open field test

The open field consists of a wooden box $90\text{cm} \times 90\text{cm} \times 38\text{cm}$, positioned in a dimly-lit room. The walls and floor are painted black. Following 2 min habituation period for the testing room, rats were placed at a corner of the open field for 5 min of free exploration. Distance moved in the central and peripheral areas of the box, as well as duration of stay, were recorded using Ethovision XT 8.

The elevated plus maze test

This maze consists of a plus-shaped platform with two open arms and two closed arms surrounded by 38-cm high opaque walls on three sides, with arms of the same type located opposite each other (File, 1993). Following 2 min habituation to the testing room, each rat was placed on the central-platform facing an open arm and was allowed to explore the maze for 5 min. Distance moved and duration of stay in open and closed arms were recorded using Ethovision XT 8.

Acoustic startle testing

Unconditioned startle response to an acoustic stimulus was measured using a standard startle chamber (Panlab SLU, Spain). Rats were held using a plastic cylinder in the startle apparatus. The apparatus is equipped with a speaker for producing sound bursts and with a highly sensitive weight transducer system that allows recording and analysis of the signal generated by the rat movement during each sound burst. Output from the transducer is led to a computer for sampling.

The protocol was adapted from Adamec et al. (2012). Prior to startle testing, animals were acclimatized to the darkened apparatus for 5 min with a background white noise level of 60 dB. Following acclimation, rats received a 50 ms burst of 120 dB rising out of the 60 dB background noise once every 30 s for 15 min. The 30 trials were conducted in the dark.

Statistical Analysis

Differences were determined using one way analysis of variance (ANOVA) or repeated measures ANOVA.

Results

No significant differences were found between groups, in none of the long term behavioral measures, as shown in figures 2, 3 & 4.

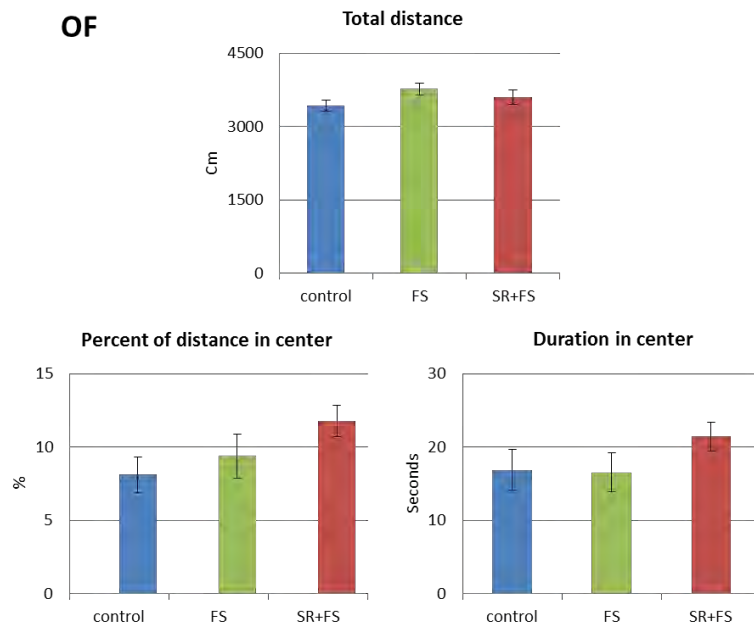


Figure 2: Open field test results. No significant differences between groups. [N: control – 8, FS – 8, SR+FS – 8]

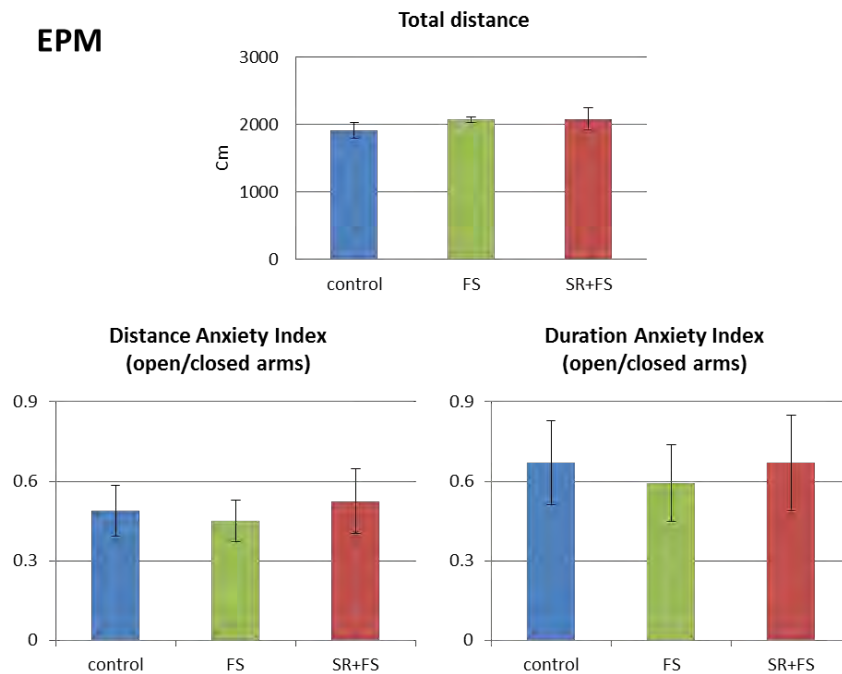


Figure 3: Elevated plus maze test results. No significant differences between groups. [N: control – 8, FS – 8, SR+FS – 8]

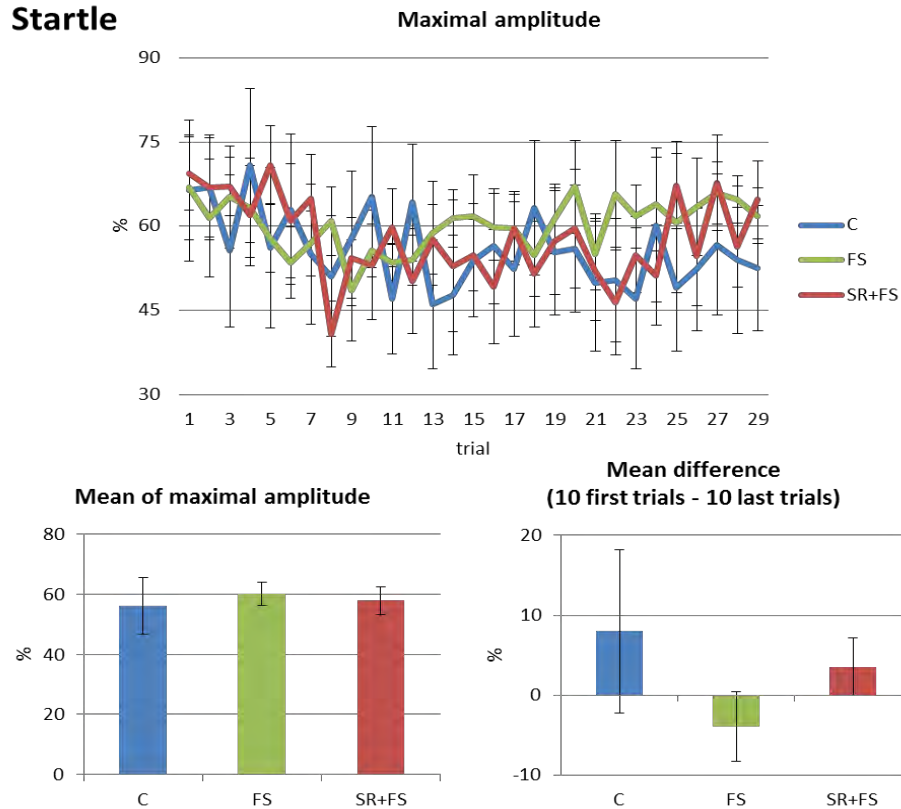


Figure 4: Acoustic startle test results. No significant differences between groups. [N: control – 8, FS – 8, SR+FS - 8]

In the acoustic startle test, as shown in figure 4, rats that were stressed with FS show no habituation what so ever in the acoustic startle response, while the rats that were sleep restricted before the FS actually tend to look more similar to the controls. The control group showed only some habituation to the sound bursts. It is possible that the animals did not habituate because this was the third test on the same day. We planned all behavioral tests on the same day, because we wanted to examine the effect of SR on the short term reaction to FS.

To summarize sleep restriction in our model does not exacerbate the short term reaction to the mild stressor of forced swim.

Tasks 3+5: Behavioral and neurobiological investigation of the impact of the under-water trauma (UWT) + The impact of pre-exposure to juvenile stress on the outcome of exposure to UWT –

In marked contrast to the lack of effect of proximal sleep restriction on the long-term outcome of exposure to a traumatic event, results regarding childhood exposure to stressors as a risk factor which exacerbates the long-term outcome of exposure to a traumatic event are clear cut. In a series of experiments we were able to demonstrate the impact of this long-term risk factor on PTSD.

In addition, we have developed a unique and a very effective methodology to analyze the behavioral outcome of the exposure to the risk factor and to the trauma at a more individual level, enabling us to identify and separate between exposed – affected individuals and exposed – non-affected individuals. This methodology enables us now to run target-expression studies in which we differentiate between these two groups. By this we are now able to identify molecular targets associated with vulnerability, with the pathology, but also with stress resilience.

We have launched a series of large scale experiments in order to benefit from the PTSD platform we have established and the analysis methodology we have developed. These experiments are conducted with a dual aim:

1. To identify novel targets for drug treatment and drug development.
2. To gain better understanding of the neurobiology of vulnerability, PTSD pathology, and of stress resilience.

To that end, and based on emerging findings we also develop in collaboration with colleagues viral vector tools to specifically and selectively modulate the expression of specific target molecules.

A Novel Approach for PTSD Modeling in Rats - Alternating Patterns of Limbic Activity in Different Types of Stress Reaction

Human reactions to trauma exposure are extremely diverse, with some individuals exhibiting only time-limited distress and others qualifying for posttraumatic stress disorder diagnosis (PTSD). Furthermore, whereas most PTSD patients mainly display fear-based symptoms, a minority of patients displays a co-morbid anhedonic phenotype. We employed an individual profiling approach to model these intriguing facets of the psychiatric condition in underwater-trauma exposed rats. Based on long-term assessments of anxiety-like and anhedonic behavior, our analysis uncovered 3 separate subtypes of stress response; an anxious, fear-based phenotype (38%), a co-morbid, fear-anhedonic phenotype (15%), and an exposed-unaffected group (47%). Combining immunohistochemical assessments for cellular activation (c-Fos) and activation of inhibition (c-Fos+GAD67) revealed a differential involvement of emotion processing regions and distinct limbic activity patterns in these different types of response.

Results

Underwater trauma affects long-term reactions to contextual reminders

To evaluate the long term effects of extreme stress exposure we employed a contextual fear conditioning protocol using the water associated zero maze model (WAZM; Ritov and Richter-levin, 2014). Following 4 days of habituation to the maze, test rats (UWT) were exposed to an underwater trauma stress in the aquatic center of the WAZM (Figure 1). No differences were found between the behavior of control and UWT rats before this pairing. One month after the conditioning, all rats were re-exposed to the WAZM test as a contextual reminder. Analyses of rats' fear response during this contextual re-exposure revealed significant differences between the control and UWT groups in time spent in the open arms [$t_{(44)}=2.4$, $p<0.05$; Figure 2B], distance traveled in the open arms [$t_{(44)}=2.4$, $p<0.05$; Figure 2C] and total freezing [$t_{(44)}=3.5$, $p<0.01$; Figure 2E]. In regard to the safer areas of the maze, control and UWT rats did not differ significantly in the distance traveled in the closed arms [$t_{(44)}=1.7$, $p>0.11$; Figure 2D]. Hedonic behavior over a long period of time was analyzed using the continuous saccharine preference test. Assessment of average saccharin preference during the 4 weeks interval between the UWT and context re-exposure revealed no significant between-group effects [$t_{(44)}=0.13$, $p>0.9$; Figure 2F].



Figure 1: The Water Associated Zero Maze
(Ritov and Richter-levin, 2014).

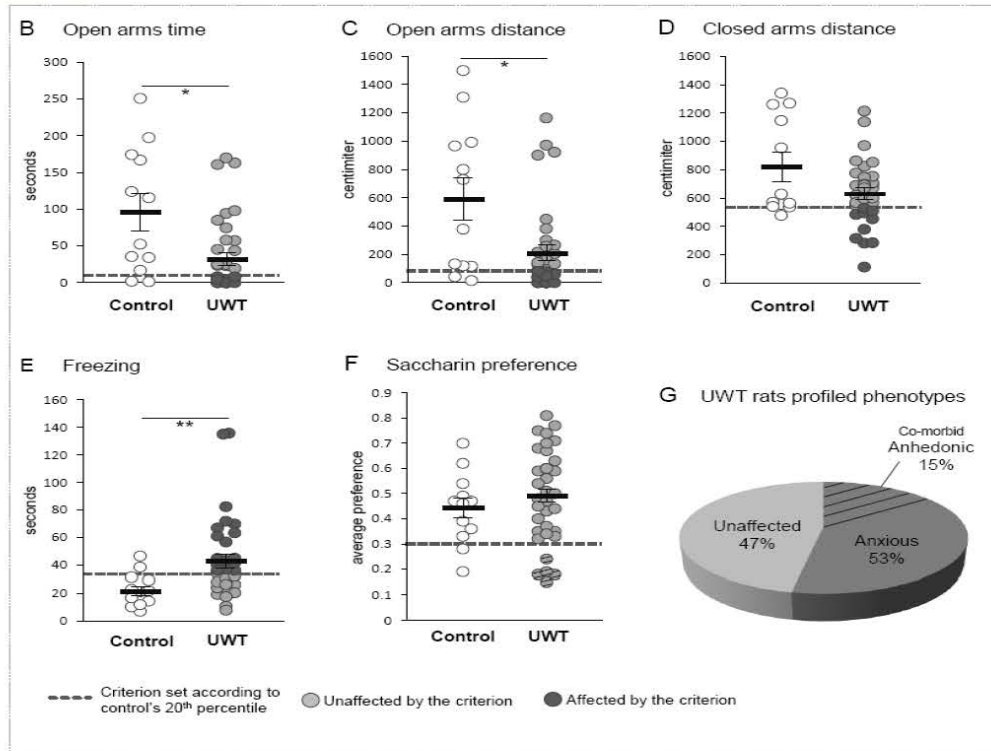


Figure 2: One month after conditioning, averaged reactions to contextual reminders of underwater trauma are increased, yet a profiling approach reveals a fundamental diversity in the individual responses of exposed rats.

A. Timeline of the experiment.

B. Re-exposure to the WAZM test as a contextual reminder of UWT extensively affects anxious-like behavior. UWT rats (n=34) spent significantly less time and travelled less distance (**C**) in the open arms of the maze, yet did not differ significantly from control rats (n=12) in the distance traveled in the closed arms (**D**). **E.** UWT rats spent significantly more time freezing during the WAZM re-exposure. **F.** UWT rats did not differ significantly from control rats in the average preference of Saccharin. Black bars represent the groups mean \pm SEM; *p<0.05; **p<0.01.

G. Individual profiling was conducted using control's group distribution. The profiling criteria based on the 20th percentile (dashed gray line) of both anxious-like behaviors (a minimum of 3 affected criteria in **B**, **C**, **D** or **E**) and anhedonia assessment (**F**). The combined profiling classification identified 2 different categories of affected style among UWT rats, affected Anxious (n=13), affected Anhedonic (n=5) and an additional exposed-unaffected group (n=16).

WAZM- water associated zero maze; **UWT**- underwater trauma; **CSP**- continuance saccharin preference assessment.

Individual profiling classification reveals different subtypes of stress response in rats

In order to profile altered behaviors within the variability of the study population, anxious-like behavior in the WAZM and hedonic behavior in the CSP were categorized according to the control group distribution (Horovitz et al, 2012). Determination of the classification criteria based on the lower 20th percentiles of control's distribution for time spent in the open arms (<10.88 sec), distance traveled in the open arms (<86.9 cm) and distance traveled in the closed arms (<539 cm) during the context re-exposure, as well as average saccharin preference in the CSP (<0.3 ratio). For determination of the freezing criterion, the classification based on the upper 20th percentile of control's distribution (>34.4 sec). Rats were then individually discerned for each of the single criterion (Figure 1B-F). For anxious-like behavior classification, every rat that demonstrated a behavior profile that falls within a minimum of 3 out of 4 criteria of fear behaviors in the WAZM was classified as an Affected Anxious (n=18; Figure 1G). Rats were then discerned for anhedonic behavior. Every individual rat which demonstrated a behavior that does not cross the criteria for average saccharin preference in the CSP (i.e. <0.3 ratio) got an additional score for being anhedonic (Figure 1F). The final classification combined the anxious-like and anhedonic profiling by discerning every individual rat for falling within the criteria of either Affected Anxious alone, anhedonic alone or both. As depicted in figure 1G, this final classification revealed 2 different subtypes of affected style among rats exposed to underwater trauma; a fear-based affected Anxious phenotype, a co-morbid affected Anhedonic phenotype, and an additional exposed-unaaffected group.

Altered activation of emotion processing regions in different subtypes of affected style

We initially assessed the activation in the sub-regions of the mPFC, NA, amygdala, hippocampus and PAG (Figure 3A), in the different subtypes of affected style. Immunohistochemical assessment of c-Fos expression as a marker of cellular activity, and GAD67 dual-labeling as a marker for active inhibitory GABAergic cells, was used to calculate regional activation (total c-Fos expressing cells) and activation of inhibition (total GAD67 labeled cells expressing c-Fos; Figure 3B). The results of this assessment indicated that the classification of rats to different affected behavioral style has functional correlates of activation and inhibition in the limbic system. One-way ANOVA's found significant effects between the response subtypes in UWT and control rats in the Prelimbic [PL; c-Fos: $F_{(3,38)}=5.4$, $p=0.003$; DL: $F_{(3,38)}=5.4$, $p<0.001$] and Infralimbic [IL; c-Fos: $F_{(3,38)}=8.9$, $p<0.001$; DL: $F_{(3,38)}=4.7$, $p=0.007$] divisions of the mPFC (Figure 3C), central [CeA; c-Fos: $F_{(3,38)}=16.3$, $p<0.001$; DL: $F_{(3,38)}=2.6$, $p=0.064$] and basolateral [BLA; c-Fos: $F_{(3,38)}=6$, $p=0.002$; DL: $F_{(3,38)}=6.8$, $p=0.001$] nuclei of the amygdala (Figure 3B). As can be seen in

figure 4, significant group effects were found in the dorsal hippocampus CA3 [dCA3; c-Fos: $F_{(3,38)}=8.5$, $p<0.001$; DL: $F_{(3,38)}=2.9$, $p=0.050$] and DG [dDG; c-Fos: $F_{(3,38)}=3.8$, $p=0.019$; DL: $F_{(3,38)}=5.9$, $p=0.002$] layers (Figure 4A), as well as in its ventral CA1 [vCA1; c-Fos: $F_{(3,38)}=5.9$, $p=0.002$; DL: $F_{(3,37)}=13.4$, $p<0.001$], CA3 [vCA3; c-Fos: $F_{(3,37)}=5.5$, $p=0.003$; DL: $F_{(3,37)}<1$] and DG [vDG; c-Fos: $F_{(3,38)}=15.6$, $p<0.001$; DL: $F_{(3,38)}=6.9$, $p=0.001$] layers (Figure 4A). Finally, significant effects between the subtypes of response and control rats were found in the Nucleus Accumbens core [NAc; c-Fos: $F_{(3,38)}=5.9$, $p=0.002$; DL: $F_{(3,38)}=1.9$, $p=0.148$] and shell [NAS; c-Fos: $F_{(3,38)}=8.1$, $p<0.001$; DL: $F_{(3,38)}=6.8$, $p=0.001$] (figure 5A), as well as in the PAG's dorsal [dPAG; c-Fos: $F_{(3,38)}=9.6$, $p<0.001$; DL: $F_{(3,38)}<1$] and ventral [vPAG; c-Fos: $F_{(3,38)}=15.1$, $p<0.001$; DL: $F_{(3,38)}=1.2$, $p=0.332$] divisions (figure 5B).

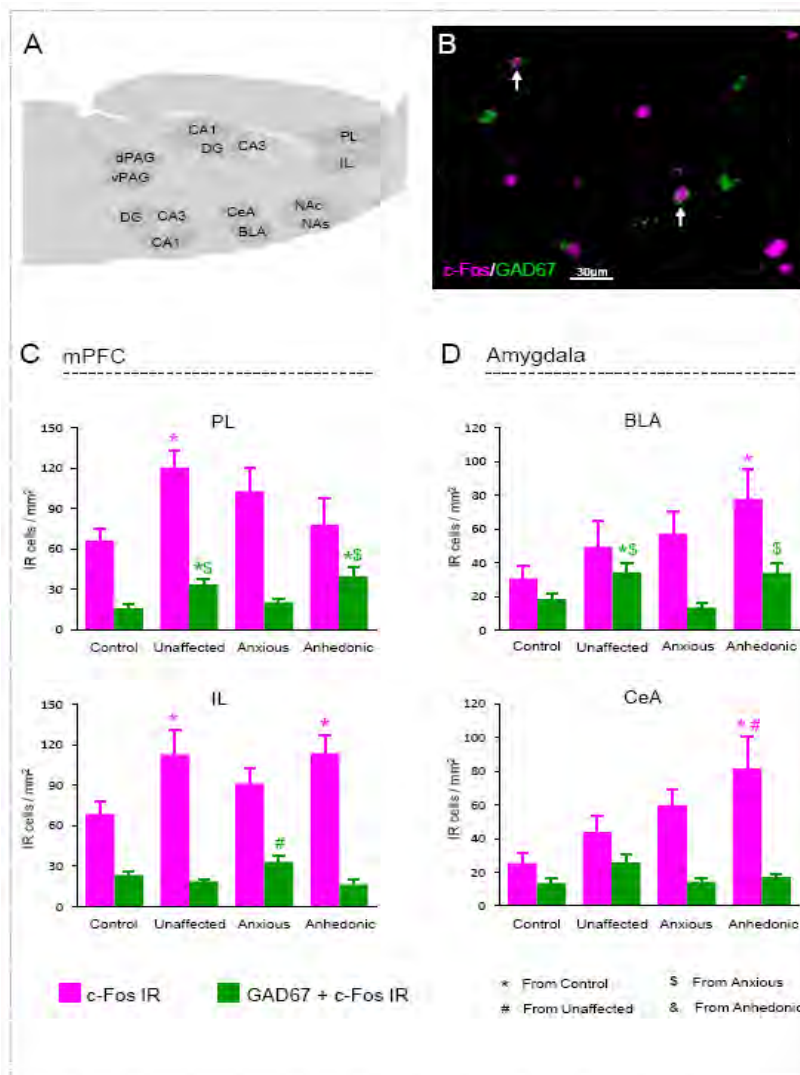


Figure 3: Differential activation of mPFC and amygdala in the different phenotypes of UWT and control rats.

A. Diagram of analyzed regions. Labeled cells were quantified bilaterally and averaged from 3 x 30µm sections per region. PL. perilimbic cortex; IL. infralimbic cortex; NAc. nucleus accumbens core; NAs. nucleus accumbens shell; BLA. basolateral amygdala; CeA. central amygdala; d/vCA1. dorsal/ventral cornus ammonis 1; d/vCA3. dorsal/ventral cornus ammonis 3; d/vDG. dorsal/ventral dentate gyrus; d/vPAG. dorsal/ventral periaqueductal gray. **B.** An example of dual-colored immunohistochemical labeling for c-Fos expression (magenta) as a biochemical marker of cellular activation and GAD67 (green) as a biochemical marker of inhibitory GABAergic cells in the BLA. White arrows point to dual-labeled cells (DL), co-expressing GAD67 and c-Fos, which is considered as activation of inhibition.

C. Differences in activation (total c-Fos expressing cells) and activation of inhibition (DL cells count) in the sub divisions of the mPFC between the different phenotypes of UWT and control rats. **D.** Differences in activation and activation of inhibition in the BLA and CeA nuclei's of the amygdala between the different phenotypes of UWT and control rats. Significant Bonferroni post-hoc results with $p < 0.05$ are flagged as: * different from Control; # different from Unaffected; \$ different from affected Anxious; & different from affected Anhedonic.

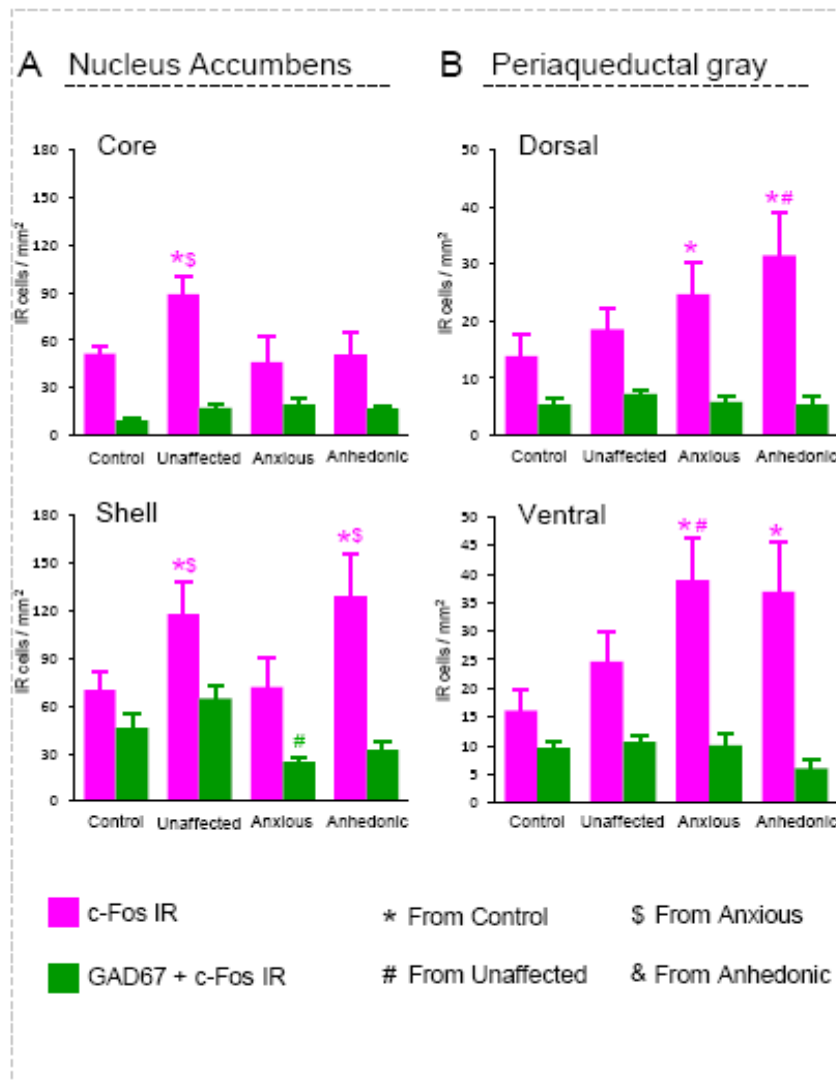


Figure 5: Differential activation in the PAG and NA in the different phenotypes of UWT and control rats.

A. Differences in activation (total c-Fos expressing cells) and activation of inhibition (DL cells count) in the nucleus accumbens layers between the different phenotypes of UWT and control rats. **D.** Differences in activation and activation of inhibition in the dorsal and ventral regions of the periaqueductal gray between the different phenotypes of UWT and control rats. Significant Bonferroni post-hoc results with $p < 0.05$ are flagged as: * different from Control; # different from Unaffected; \$ different from affected Anxious; & different from affected Anhedonic.

Limbic network patterns of activity shift in accordance to the behavioral response

In order to test the hypothesized contribution of differential modulation within the mPFC-hippocampus-amygdala circuit to the diversity of stress response (Lanius et al, 2010), we implemented further analyses to characterize the network pattern of activity in each behavioral subtype. At the first step, we depicted the significant alterations in inhibitory activation at the network level. Every DL count that significantly differed from control was normalized to the relevant control's group mean (i.e. individual DL count - control mean DL count / overall standard deviation). As shown in figure 6A, the unaffected type of response associated with an increase of inhibitory activity in the PL, BLA, dCA3 and vDG. In contrast to that, no substantial change was observed in the inhibitory pattern of the affected phenotypes. The anxious type of response associated with a decrease of inhibition only in the vCA1 (Figure 6B), while the co-morbid anhedonic type associated with an increased inhibition in the PL and dCA3 (Figure 6C).

At the next step, we implemented further analyses to explore the patterns of excitation that associate to the different responses. In order to isolate the excitatory projections from the overall activation, initial excitation scores for each region were calculated by reducing the counts of activated GABAergic cells (DL count) from the total count of c-Fos expressing cells. Following this, the significant effects of response type on regional excitation were screened using one-way ANOVAs with Bonferroni post-hoc correction. Only regional excitations, of UWT rats, that significantly differed from the control's group mean were used for further analysis. Relevant scores were normalized to the control group mean (i.e. individual score - control mean score / overall standard deviation) and clustered to illustrate major alterations in patterns of excitatory activity. In contrast to the patterns of inhibitory activation, this has revealed a prominent shift in the excitation patterns of the affected phenotypes (Figure 6). While the pattern of unaffected rats was rather similar to control (Figure 6D), the affected phenotypes patterns involved a general increase in the excitation of the amygdala, ventral hippocampus and PAG. Furthermore, in the anxious phenotype, a distinct increase was found in both ventral and dorsal CA3, accompanied with a distinct decrease in dDG excitation (Figure 6E). In the anhedonic phenotype, a distinct increase was found in the NAs excitation (Figure 6F).

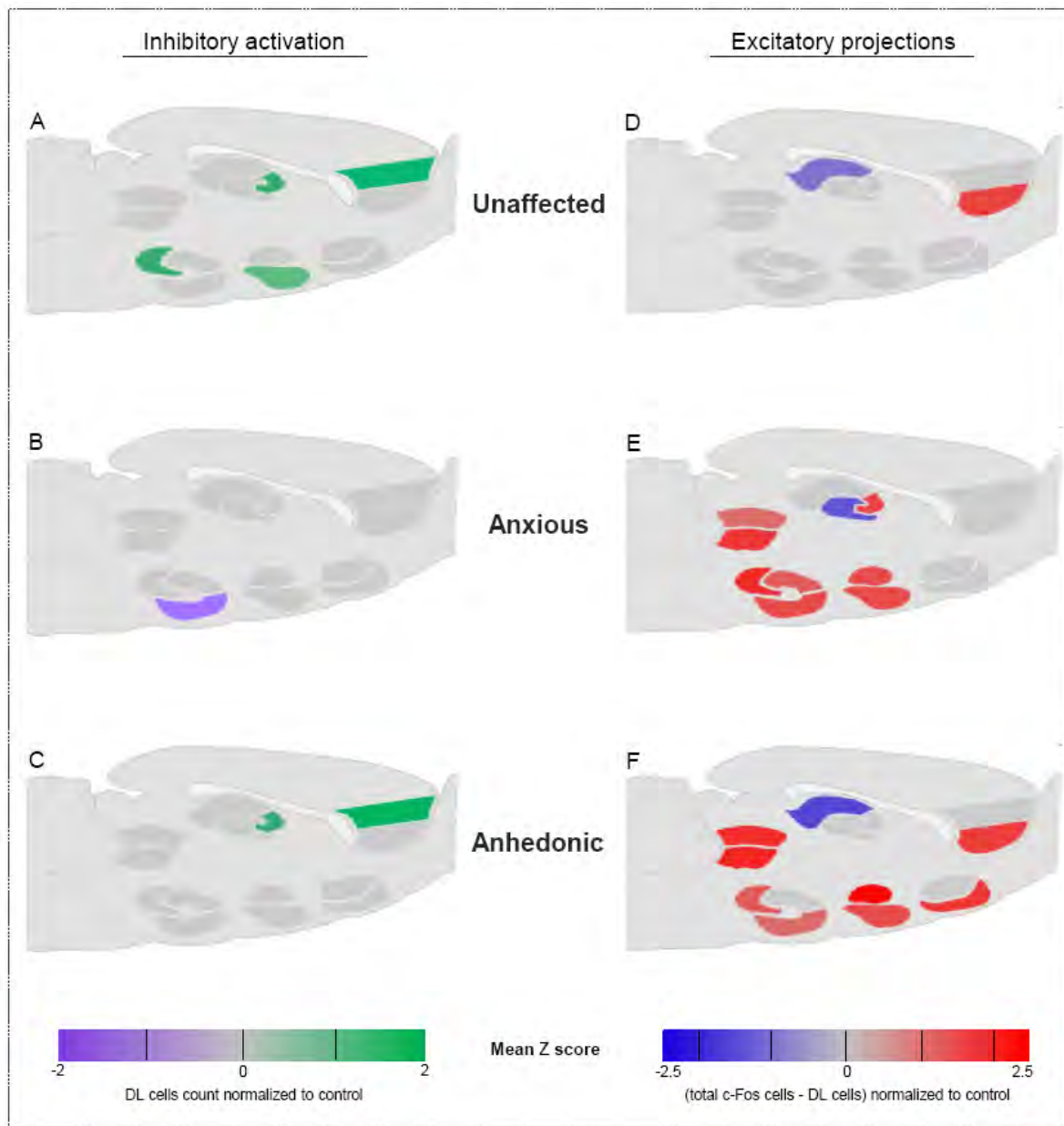


Figure 6: Limbic patterns of activity shift in accordance to the behavioral response of UWT rats.

Left panel- significant alterations in inhibitory activation [DL count normalized to control] at the network level. Right panel- significant alterations in excitatory projections [(c-Fos count - DL count) normalized to control] at the network level. When compared to control (Gray), the unaffected rats, that went through the UWT but did not have an anxious-like response during the context re-exposure, show a network pattern of increased inhibition in the dCA3, vDG, BLA and PL (**A**), with no major enhancement of excitation (**D**). In contrast, the affected anxious rats show a network pattern of decrease inhibition in the vCA1 (**B**) and a prominent enhancement of excitation in the amygdala, ventral hippocampus and PAG (**E**). The co-morbid anhedonic rats show a network pattern of no major enhancement of inhibition (**C**) and a prominent enhancement of excitation in the amygdala, ventral hippocampus, PAG and NAs (**F**).

Discussion

It was previously shown that a single episode of acute underwater stress increases anxious-like behaviors and differentially affects the dorsal and ventral layers of the hippocampus, both at the short (Ritov et al, 2014) and long terms (Sood et al, 2013; Sood et al, 2014). By introducing an individual profiling approach, which models the diagnostic criteria of PTSD in rats (Cohen et al, 2014; Matar et al, 2013), to underwater stress-exposed rat population, we have identified an affected, extremely anxious, sub-group with an occurrence rate of 53%. This prevalence highly resembles the probability for males to meet full PTSD diagnosis following severe trauma exposure, as combat or rape (~40-60%; Nemeroff et al, 2006). Furthermore, combining both anxious-like and anhedonic behavioral assessments enabled us to discern between different subtypes of reaction style and specify 2 phenotypes of affected rats, an utterly fear-based anxious phenotype and a co-morbid anhedonic phenotype. This diversity within affected rats responses is similar to the psychiatric acknowledged symptoms range of more anxious- fear based, or more anhedonic- dysphoric responses to trauma (APA, 2013), as well as phenotypes variety in PTSD patients (Lanius et al, 2012).

Disregarding the pattern of different symptoms, by grouping of all patients in PTSD research, has been suggested to limit the understanding of posttraumatic psychopathology and its neural correlates (Lanius et al, 2010). The significant differences we found between the different subtypes of stress response, and control rats, in activation and inhibition of different regions further validates the individual profiling approach and the functionality of its behavioral classifications. Combining this classification with the characterization of the network pattern of excitation revealed a fundamental difference between the excitation patterns of the unaffected rats and the affected anxious or anhedonic phenotypes. Thus, allowing us to characterize the neural systems that correlate with each affected phenotype. Among all affected rats, a significant increase of excitation was observed in the amygdala, ventral hippocampus and PAG. This is in line with previous suggestions regarding stress effects on the ventral hippocampus (Maggio and Segal, 2012; Segal et al, 2010) and its involvement in long term anxious reactions to underwater trauma (Sood et al, 2013; Sood et al, 2014). Nevertheless, notable excitations, differentiating between the more anxious and anhedonic phenotypes, were observed in the dorsal hippocampus, NA and mPFC. The more anxious phenotype associated with a distinct decrease of dorsal DG excitation and an increase of dorsal CA3 excitation (Figure 6E). In contrast, the anhedonic phenotype associated with a distinct increase of NAs excitation (Figure 6F).

Since the co-morbid group, classified as anhedonic, was rather small (n=5) in this study, any conclusions regarding its neural correlates should be treated with caution. Nevertheless, the

occurrence rate of this condition among the overall affected anxious rats in our study was 27.8%. This is similar to the reported rate of co-morbid major depression occurrence (23%) among PTSD patients (Conner et al, 2014). As in the clear anxious phenotype, the anhedonic rats showed an increase of amygdala and ventral hippocampus excitation. However, this co-morbid condition additionally associated with an increase of excitation in the IL (Figure 6F), as was also found in the unaffected group (Figure 6D). This is line with human imaging data, indicating that following trauma exposure both unaffected individuals and PTSD patients with co-morbid major depression respond to trauma reminders with grater mPFC activation in comparison to clear PTSD patients (Lanius et al, 2007). These anhedonic rats also showed a prominent increase of excitation in the NAs, a key brain region for hedonic behavior (Berridge and Kringelbach, 2013).

When taken together, these conclusions strongly support the use of individual profiling approaches for discerning affected sub-groups among the populations of studies using rodent models of PTSD. The combination of behavioral profiling and network level analyses may therefore be used to better model the human condition. Accurately identifying vulnerable individuals, and different affected styles among stress exposed rodents, can promote the development of precision interventions that target specific behavioral and neuronal connectivity patterns. This can direct future translational research and help bridging the gap between rodent's behavior and human psychopathology.

Long-term alterations in neuropeptide gene expression in sublayers of the dorsal dentate gyrus in a rat model of Posttraumatic stress disorder

Introduction

After exposure to traumatic events only a subset of individuals develop posttraumatic stress disorder (PTSD), characterized by persistently increased anxiety, hypervigilance as well as inapt trauma-related memories and cognitive deficits (*DSM-V, APA, 2013*). Epidemiological studies suggest childhood adversity as a prominent risk factor for developing PTSD and depression later in life (*Heim & Nemeroff, 2001*) and can be modeled in rodents by variable, uncontrollable ‘juvenile stress’ (JS; *Horovitz et al., 2012*).

In this study we now combined JS with a brief, traumatic submission under water (underwater trauma, UWT), that has been shown to increase anxiety and alter plasticity and local circuit activity in the dorsal dentate gyrus (DG) in rats (*Richter-Levin, 1998; Wang et al., 2000; Ardi et al., 2014*). Since information flow in the DG is shaped by local inhibition via different interneuron subpopulations (*Houser, 2007; Coulter & Carlson, 2007*), we analyzed in a lasting mRNA expression changes of the distinct interneuron markers GAD65 and GAD67 as well as the neuropeptides cholecystokinin (CCK), neuropeptide Y (NPY) and somatostatin (SST) in dorsal dentate gyrus (dDG) sublayers as well as in the basolateral complex of the amygdala (BLA).

Moreover, individual behavioral profiles were compiled based on the behavioral performance in the elevated plus maze (EPM) to identify ‘emotionally affected’ individual animals.

This approach revealed an increased expression of CCK and also NPY in the dDG granule cell layer of affected animals.

Methods

Behavior:

groups	JS	UWT + odor reminder	EPM after odor reminder	Brain Prep
<i>PND</i>	<i>27-28</i>	<i>60</i>	<i>90</i>	<i>90</i>
Control	-	-	+	+
UWT(+)	-	+	+	+
JS+UWT(+)	+	+	+	+

Juvenile stress (JS): 3 consecutive days of stress exposure:

PND27: Forced swim stress (10 min, water temp. $22\pm 2^{\circ}\text{C}$)

PND28: Elevated platform (3x 30 min; 60 min ITI in home cage)

PND29: 2 h restrain.

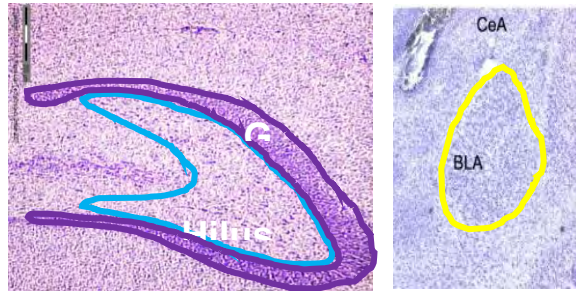
Underwater trauma (UWT) + odor:

3 days habituation to standard cage with bedding (2 min each), Day 4: Exposure to vanilla odor in cage (3 air puffs, 10s ITI, after 2 min habituation), then immediately placed in water tank (50x60x60 cm), Restraint under water after 5 s swim for 45s with a special metal net.

Elevated plus maze test (EPM) + odor reminder: Vanilla odor re-exposure (3 air puffs, 10s ITI, after 2 min habituation), then immediately placed on EPM arena (110 x 110 cm, 70 cm above floor; two opposing open/closed arms, with 35 cm high walls, no roof; full light) 5 min free exploration, recorded and analyzed by EthoVision XT8 tracking system.

Laser Capture Microdissection & RNA- Isolation

02µm thick coronar sections at the level of dorsal hippocampus (Cryostat) on Poly-L-Lysine coated RNase free PEN Membran slides (PALM). Brief staining with 1% Cresyl violet acetate. Identification of targetregions (5x magnification): dorsal DG – granule cell layer & hilus, basolateral complex of the amygdala (BLA). laser microdissection (Leica systems). Isolation of total RNA with QIAGEN RNeasy Micro Plus Kit.



Quantitative real-time PCR

- Reverse transcription of RNA (QIAGEN Sensiscript kit; Random Decamer & Oligo dT-Primer)
- multiplex real time PCR: TaqMan- assays (Applied Biosystems) for target genes and the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for triplets of each sample
- ddCT Method (Livak & Schmittgen, 2001): Determination of cycle thresholds (CT) for each sample, normalized to the expression of the housekeeping gene GAPDH:

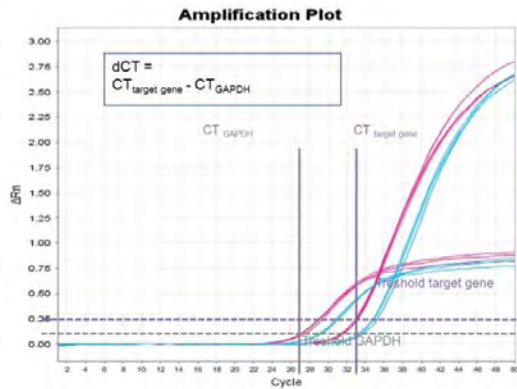
$$(1) \text{dCT}_{\text{sample X}} = \text{CT}_{\text{target gene}} - \text{CT}_{\text{GAPDH}}$$

- Normalization towards mean dCT of all control samples for each sample (region- and target-specific) :

$$(2) \text{ddCT}_{\text{sample X}} = \text{dCT}_{\text{sample X}} - \text{mean dCT}_{\text{control}}$$

- Transformation of ddCT relative quantification value (RQ), comparing expression levels towards the control group within a specific region and for a specific target gene ($\text{RQ}_{\text{control}} = 100\%$):

$$(3) \text{RQ}_{\text{sample X}} = (2^{-\text{ddCT}_{\text{sample X}}}) * 100 (\%).$$



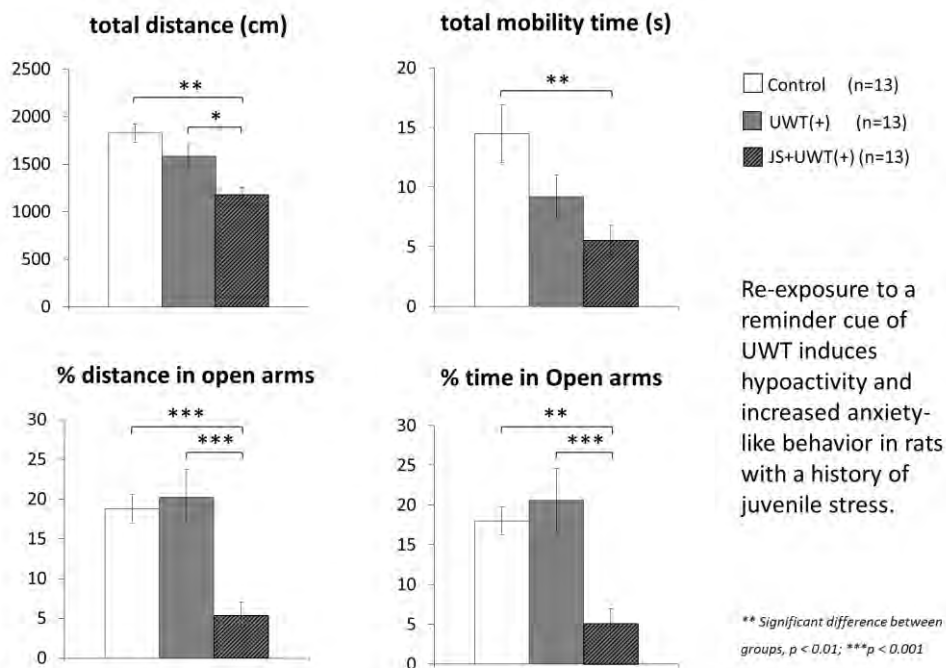
Statistical analysis:

- Shapiro-Wilks test for normality
- Effects of group: One Way ANOVA followed by LSD post hoc test or Kruskal-Wallis-Test.
- Distribution of affected vs. unaffected populations: Pearson's chi-squared test
- Comparison of profiles: Paired Samples t-test or Mann-Whitney U-test.

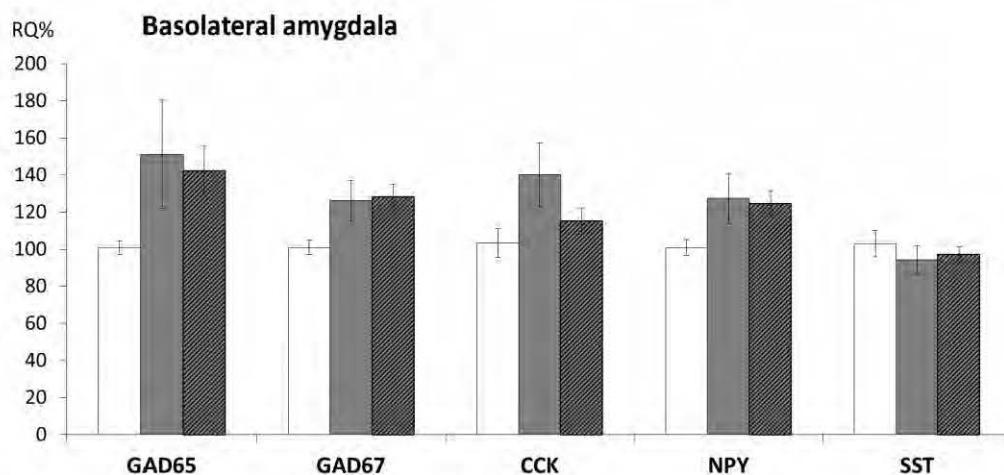
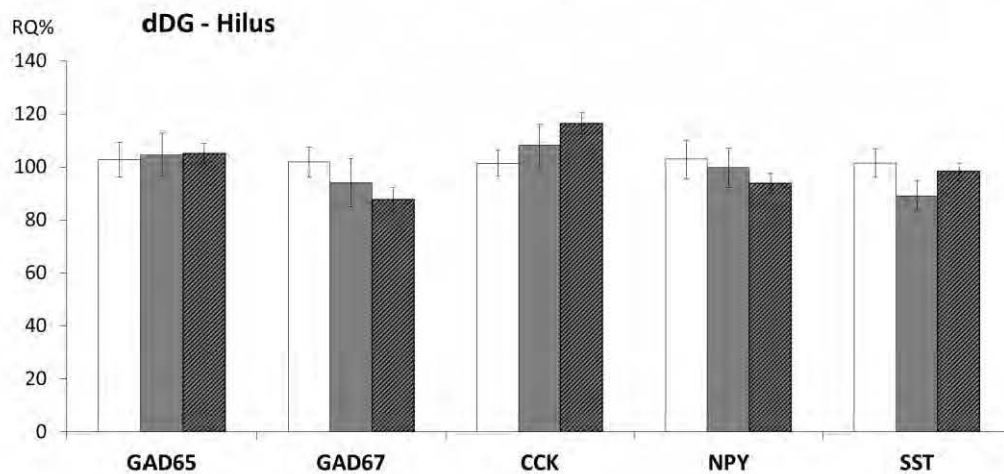
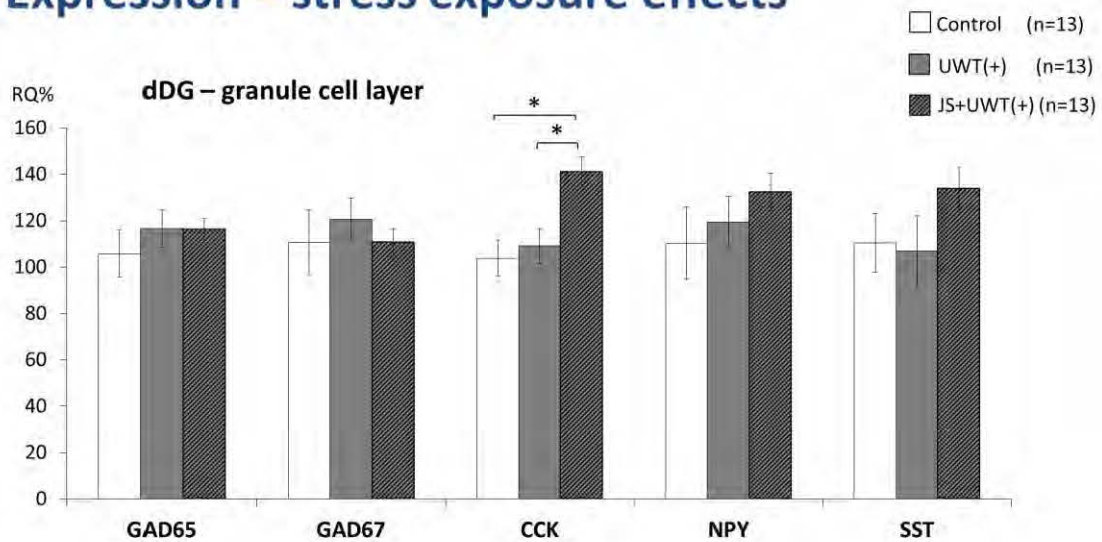
All tests using SPSS 19 software

Results

Behavior – stress exposure effects



Expression – stress exposure effects



Exposure to combined juvenile stress and UWT is associated with a long-lasting increase of CCK mRNA expression levels in the dDG granule cell layer.

No significant expression changes were observed, neither for any other observed target gene nor in the Hilus of the dorsal DG or the basolateral complex of the amygdala (BLA).

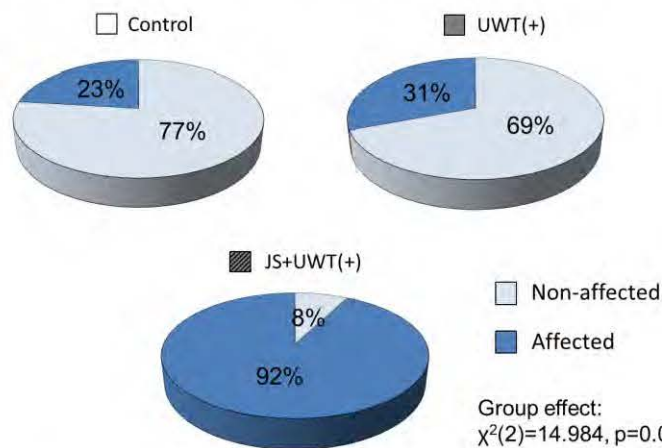
* Significant difference between groups, $p < 0.05$

Behavioral profiling

TOTAL	TOTAL	% OA	distance	time
Distance	Mobility			
1604.8878	8.8400	14.7373	13.8400	
1975.7631	17.2200	21.5359	21.2400	
2033.845	18.12	20.31126	20.16	
1660.692	11.76	17.306021	8.839999	
1843.553	16.32	24.56117	21.92	
1898.626	15.6	20.64906	25.72	
2013.021	20.8	20.30734	21.44	
1598.776	7.68	22.10436	18.52	
1999.434	18.6	15.29432	11.24	
1952.104	14.76	19.19172	17.8	
1841.926	10.92	17.54527	16.16	
1350.84	5.04	16.2202	15.84	
1611.22	6.96	21.64155	18.88	
1558.98	10.2	26.79613	20.04	
1615.31	12.24	7.017972	8.84	
1987.488	14.04	32.16731	17.1	
1911.752	13.08	28.02817	27.1	
1489.092	12.36	20.49984	11.76	
658.7245	0	0	0	
1332.157	3.36	0	0	
1310.582	4.68	7.267737	6.24	
1330.109	4.44	27.14820	22.48	
1675.169	7.2	8.15695	7.44	
1126.84	1.68	22.46672	23.28	
1535.06	14.04	17.97658	22.52	
2016.75	11.88	29.80478	29.76	
1481.46	23.52	17.08730	43.12	
1736.5	9.36	21.81584	30.16	
881.084	1.08	0	0	
1851.537	15	4.167688	2.84	
870.7721	2.4	0	0	
850.5442	1.310998	0	0	
1461.688	6.96	5.658625	3.92	
864.6143	1.800001	0	0	
1841.589	13.08	12.97758	11.76	
1487.93	12.36	1.911561	2	
755.2304	1.2	0	0	
851.793	2.28	19.39168	18.68	
1185.06	4.8	12.58578	13.04	
1400.74	8.04	6.732249	6.04	
1035.52	1.8	6.506238	7.6	

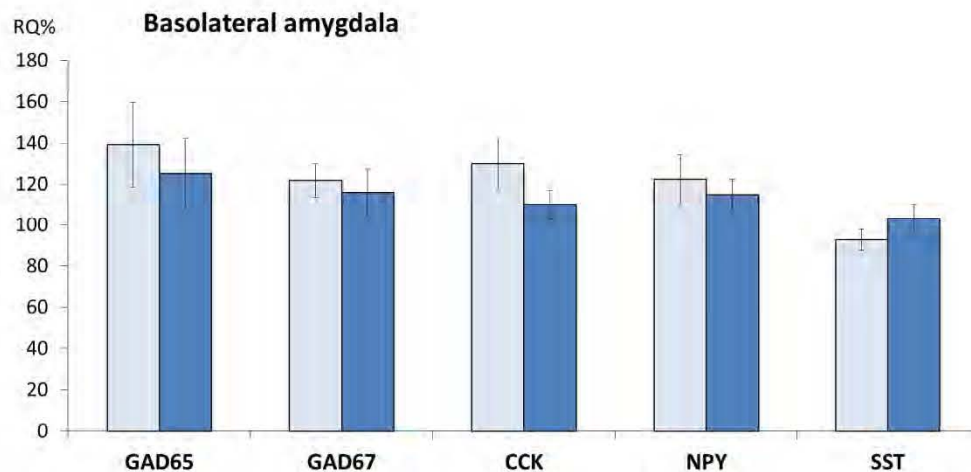
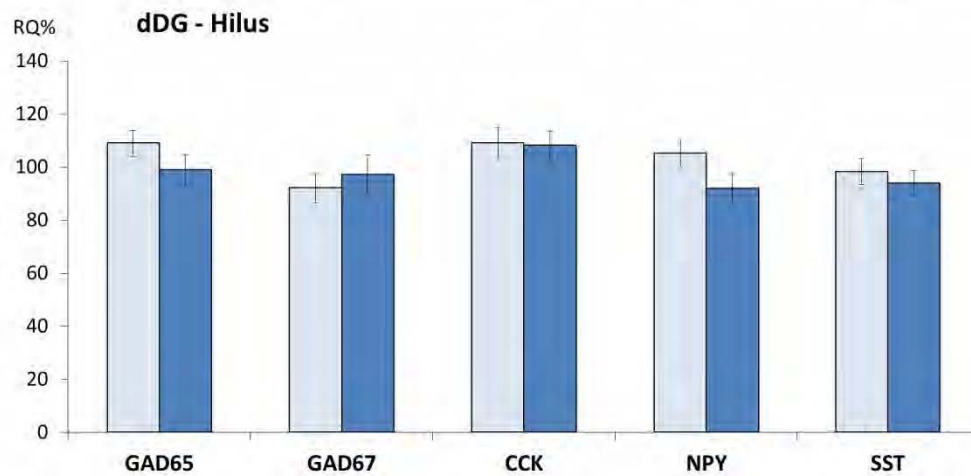
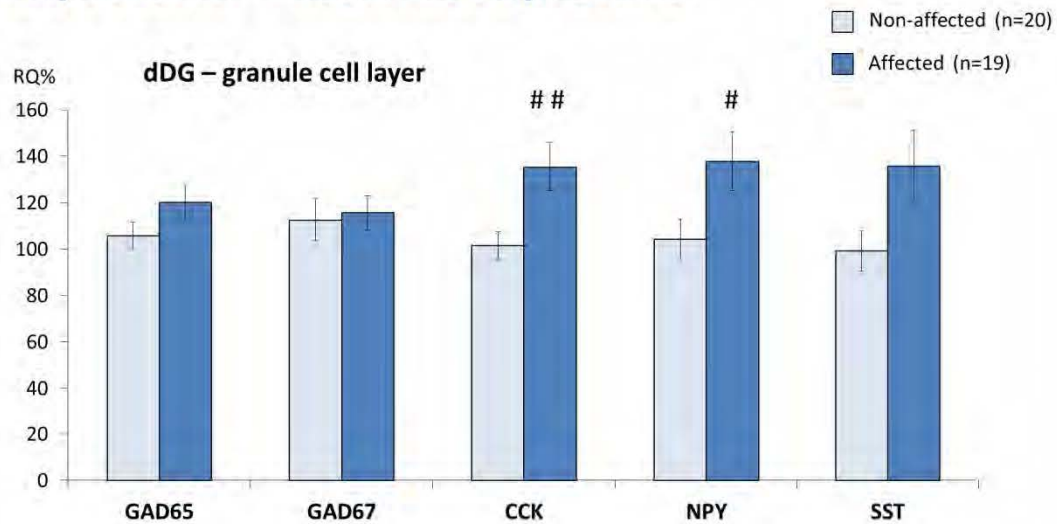
'emotionally affected' rats show an extreme anxiety-like response in the elevated pluse maze (EPM):

■ < 25 % of Control group ■ > 75 % of Control group



Rats that experienced juvenile stress and UWT show a higher prevalence for being affected

Expression – Individual profiles



Behavioral profiling based individual EPM results confirmed the increase CCK mRNA expression in rats that were classified as 'emotionally affected'.

Moreover, the behavioral profiling analysis approach revealed a parallel increase of NPY mRNA expression levels in affected animals in the same layer.

Significant difference unaffected vs. affected, $p < 0.05$

Summary & Conclusion

- ◆ Distinct **long-lasting increase of CCK mRNA** expression levels in the **ddDG** granule cell layer within a **rat model of PTSD**
- ◆ **Behavioral profiling** revealed that specifically in 'emotionally affected' animals with a high anxiety-like response such **CCK** upregulation is evident and furthermore paralleled by an increase of **NPY** mRNA expression



lasting impact of a history of stress on distinct interneuron subpopulations of the DG that may affect DG local circuit activity

Indeed, the effects of UWT and of JS+UWT on local circuit activity is currently being examined.

Task 7: Efficacy of intervention – SSRI's versus GABA/CRF related manipulations –

Following the establishment of the PTSD model we have started to employ it also as a drug-testing platform. The main challenge in that regard is that it is not possible to establish pharmacological validity, as is typically expected since there is no effective pharmacological treatment to PTSD which may be used as a reference point. However, because the new PTSD model holds exceptionally high face validity to PTSD, it seems valuable to examine the effectiveness of novel target drugs on PTSD symptoms in this model.

We have launched a large scale pharmacological study, in which we examine the effectiveness of several potential treatments. The choice of potential drugs is closely based on findings from task 3+5 above. It is important to note thus that progress in this task is partially dependent on progress in task 3+5.

Behavioral effects of DHEA-S on long-term effects of Underwater trauma with prior juvenile stress

In this experiment we aimed to study the therapeutic power of Dehydroepiandrosterone-Sulfate (DHEA-S), an exogenous neurosteroid, on the anxiety levels of rats that were exposed to UWT following an odor reminder with prior juvenile stress. Endogenous neurosteroids serve as modulators of the GABAergic function in the stress response. Neurosteroids are now being studied as potential possible treatment in stress-related disorders. In this experiment we hypothesized that DHEA-S might have a beneficial effect on the rats exposed to juvenile stress and UWT in our model. Rats were treated chronically with sc injections of DHEA-S once a week for 4 weeks, following the UWT and until a day before the behavioral testing (adapted from Hoffman et al, 2003 and Jacob et al, 2009).

Methods

Animals

Male Sprague Dawley rats (22 days old, 30-50 g) were used for the experiment. Animals were housed in groups of ~3, at $22 \pm 2^\circ\text{C}$ under 12-h light/dark cycles with water and food ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions.

Juvenile and UWT stress + DHEA-S [J+U+DHEA-S] – Rats were exposed to 'juvenile stress' and in adulthood to 'UWT stress'. Following UWT, they were chronically treated with DHEA-S once a week (sc injection) during 4 weeks until the behavioral testing.

Juvenile and UWT stress exposures + Vehicle [J+U] – Rats were exposed to 'juvenile stress' and in adulthood to 'UWT stress'. Following UWT, they were treated with vehicle once a week (sc injection) during 4 weeks until the behavioral testing.

UWT stress + Vehicle [U] – Rats were exposed to 'UWT stress' in adulthood, but not to 'juvenile stress'. Following UWT, they were treated with vehicle once a week (sc injection) during 4 weeks until the behavioral testing.

Juvenile stress + Vehicle [J] – Rats were exposed to 'juvenile stress', but not to 'UWT stress' in adulthood. Following UWT timing, they were treated with vehicle once a week (sc injection) during 4 weeks until the behavioral testing.

Control + Diazepam [DHEA-S] – Rats were neither exposed to 'juvenile stress' nor to 'UWT stress' in adulthood. Following UWT timing, they were chronically treated with DHEA-S once a week (sc injection) during 4 weeks until the behavioral testing.

Control + Vehicle [Control] – Rats were neither exposed to 'juvenile stress' nor to 'UWT stress' in adulthood. Following UWT timing, they were treated with vehicle once a week (sc injection) during 4 weeks until the behavioral testing.

Experimental design

The experimental design was similar to the previous experiments, only treatment groups were chronically treated with DHEA-S. Following UWT, J+U+DHEA-S and DHEA-S groups received the first sc injection of DHEA-S 10mg/kg (Sigma-Aldrich Co. LLC). 4 more injections were given to rats once a week. Last injection was given a day before the EPM test. Accordingly, all other rats received sc injections of the vehicle.

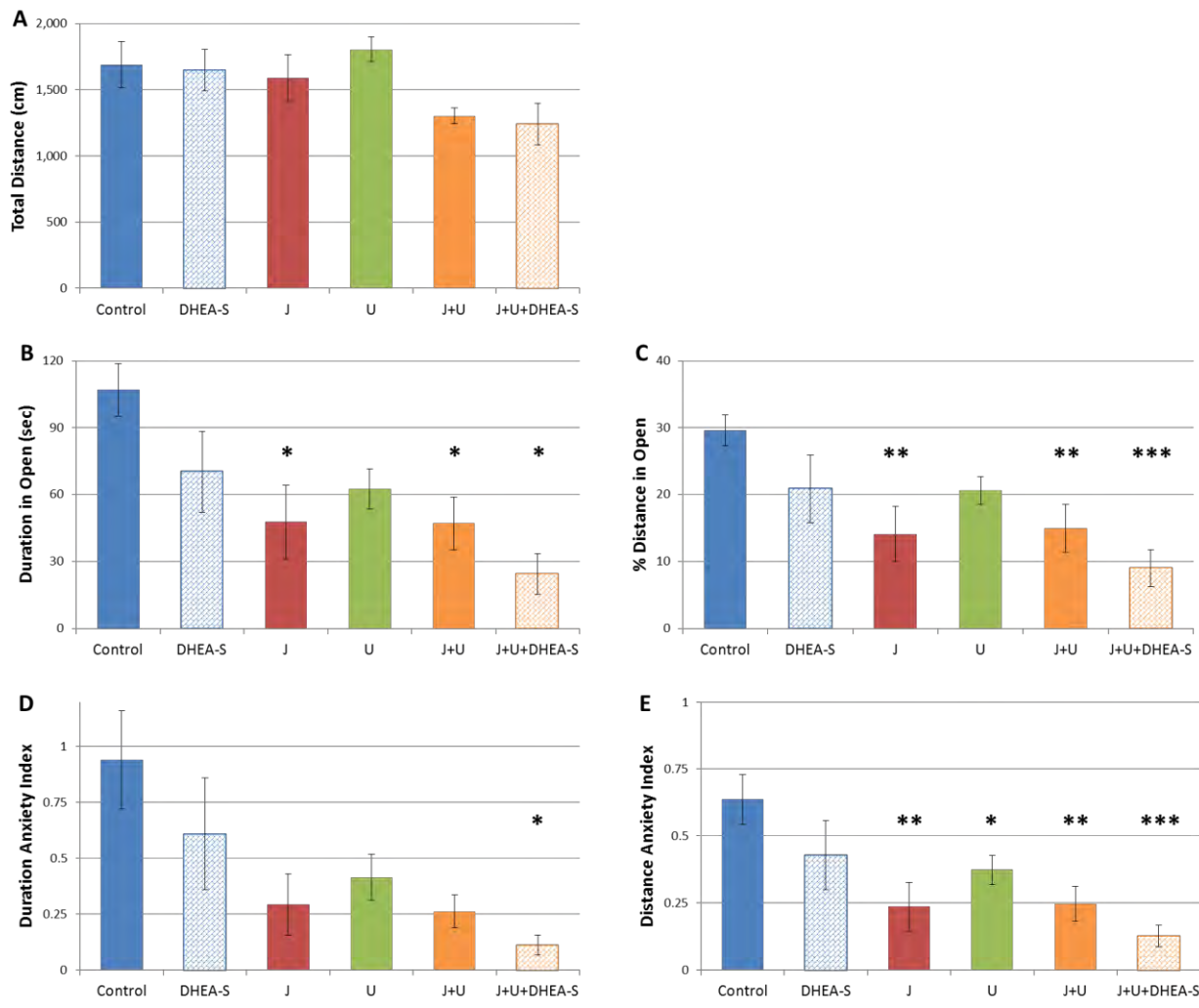
Statistical analysis.

Data was analyzed using One-way ANOVA or Independent-samples Kruskal-Wallis test depending on Shapiro-Wilks test for normal distribution. LSD post hoc comparisons were used as needed.

Results

Behavioral results of the EPM test are presented in Fig 3-1. One-way ANOVA or Kruskal-Wallis H test revealed significant differences between groups in duration of stay [$\chi^2_{(5)} = 15.31$, $p < 0.01$] and percent of distance covered in the open arms [$F_{(5,31)} = 3.82$, $p < 0.01$], as well as in both duration and anxiety indices [$\chi^2_{(5)} = 15.12$, $p = 0.01$; $F_{(5,31)} = 4.12$, $p < 0.01$], but not in total distance covered. Post-Hoc comparisons showed that juvenile stressed rats, whether or not they were also exposed to UWT, or treated with DHEA-S, spent less time and covered less distance in the open arms, and exhibited higher anxiety-like behavior according to the distance anxiety index, compared to controls ($p < 0.05$ and less). Only rats that were exposed to JVS as well as UWT and were also treated with DHEA-S, also exhibited higher anxiety-like behavior according to the duration anxiety index ($p < 0.05$). In addition, rats that were exposed to UWT alone exhibited higher anxiety-like behavior according to the distance anxiety index ($p < 0.05$).

Within rats that were exposed to JVS and UWT, there was no significant difference between rats that were treated with DHEA-S or with vehicle. In fact it seems that the DHEA-S treatment tended to increase anxiety-like behavior in the EPM within these rats. Thus we decided to stop the experiment with only 6-7 rats per group. Clearly the DHEA-S did not have a beneficial effect in our model.



Fig

3-1 Effects of exposure to JVS, UWT and DHEA-S treatment on anxiety-like behavior in the elevated plus maze. Data expressed in means±SEM. Juvenile stressed rats, whether or not they were also exposed to UWT or treated with DHEA-S, spent less time (B) and covered less distance (C) in the open arms, and exhibited higher levels of anxiety in the distance index (E), compared to controls. JVS+UWT+DHEA-S group also exhibited higher levels of anxiety in the duration index (D). Finally, UWT alone significantly increased anxiety levels only in the distance anxiety index (E). *-Compared to control, $p<.05$, **- $p<.01$, ***- $p<.001$. [N: control-6, DHEA-S-7, J-6, U-6, J+U-6, J+U+DHEA-S-6.]

Discussion

Within rats that were exposed to JVS and UWT, there was no significant difference between rats that were treated with DHEA-S or with vehicle. In fact it seems that the DHEA-S treatment tended to increase anxiety-like behavior in the EPM within these rats. Thus we decided to stop the experiment with only 6-7 rats per group. Clearly the DHEA-S did not have a beneficial effect in our model.

Behavioral effects of Fluoxetine on long-term effects of underwater trauma with prior juvenile stress

Behavioral effects of Fluoxetine during juvenility on long-term effects of underwater trauma with prior juvenile stress

In a previous experiment we found no beneficial effect of the fluoxetine (FLX) treatment on the rats' anxiety-like behavior as measured by the EPM test. In that experiment the FLX treatment was administrated during adulthood, immediately following UWT, for 4 weeks until the EPM test. We recently found in our lab that exposing rats to enriched environment during juvenility, and not during adulthood, was effective in reducing anxiety-like behavior in the long run (Ardi et al, in preparation). Thus in the current experiment we examined the possibility that the FLX may be more effective in reducing anxiety-like behavior at this time point – immediately following the JVS and until the UWT. As previously, rats received fluoxetine daily in their drinking water, but this time it was after the JVS and until the UWT, lasting almost 5 weeks. 4 weeks following UWT, rats were tested for anxiety-like behavior in the EPM test, following the odor reminder.

Methods

Animals

Male Sprague Dawley rats (22 days old, 30-50 g) were used for the experiment. Animals were housed in groups of ~3, at $22 \pm 2^{\circ}\text{C}$ under 12-h light/dark cycles with water and food ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions:

JVS+UWT+FLXpre - Rats were exposed to both JVS and UWT and treated with FLX following the JVS and until UWT.

JVS+UWT – Rats were exposed to both JVS and UWT.

Control+FLXpre - Rats were not exposed to any stress procedure and were treated with FLX since juvenility.

Control – Rats were not exposed to any stress procedure.

Experimental design

The experimental design was similar to the previous experiments, only treatment groups were treated with FLX since juvenility. Following JVS, rats in control+FLXpre and JVS+UWT+FLXpre groups were treated chronically with fluoxetine via drinking water for approximately 5 weeks until the UWT (protocol adapted from McNamara et al., 2010). 3 days prior to drug delivery, 24 h water consumption was determined for each cage. FLX daily dosage of 10 mg/kg/day was diluted in drinking water for the treated groups (as in the previous study). This dosage was selected based on previous studies demonstrating that it produces clinically-relevant plasma concentrations, reduces cortical serotonin turnover in rats, and reduces behavioral indices of depression in the forced swim test. Fresh solutions were prepared twice a week using FLX stock solution (3 mg/ml) (Vetmarket, Petah-Tikva, Israel) that was added to drinking water at the required concentration. FLX concentration was determined according to average daily fluid consumption and body weight that were measured twice or once a week, respectively. Amber opaque drinking bottles were used to protect FLX from light degradation. All other rats were receiving regular drinking water.

Statistical analysis.

Data was analyzed using Two-way ANOVA.

Results

As depicted in figure 4-1, Two-way ANOVA of stress exposure X treatment on EPM results revealed a significant main effect for stress exposure on total distance covered [$F(1,46)=14.28$, $p<0.001$], duration of stay in the Open arms [$F(1,46)=38.1$, $p<0.001$], percent of distance covered in the open arms [$F(1,46)=35.04$, $p<0.001$], as well as on both duration and distance anxiety indices [$F(1,46)=31.59$, $p<0.001$; $F(1,46)=38.9$, $p<0.001$; respectively]. Rats that were exposed to JVS and UWT, whether or not they were treated with FLX following JVS and until UWT, covered significantly less distance in the total maze, spent significantly less time and covered less distance in the open arms, and exhibited significantly higher levels of anxiety in both duration and distance indices (lower ratios indicate higher anxiety levels), compared to control rats.

There was no significant main effect for treatment on any of the measures.

There were found significant interaction effects between stress exposure and treatment on duration in the open arms [$F(1,46)=4.91$, $p<0.05$] and on both duration and distance anxiety indices [$F(1,46)=5.64$, $p<0.05$; $F(1,46)=4.76$, $p<0.05$; respectively]. Within rats that were not treated with FLX as well as within rats that were treated with FLX following JVS and until UWT, the rats that were exposed to JVS and UWT spent less time in the open arms and exhibited higher levels of anxiety-like behavior in both indices, compared to controls. This difference was larger within the treated groups. However, only in the distance anxiety index there was found a significant difference within the control rats, between rats that were treated with FLX during juvenility ($M=0.59$, $SD=0.19$) and the rats that weren't ($M=0.43$, $SD=0.2$). Apparently this treatment of FLX lowered the anxiety-like behavior according to this measure but only within control rats. No such effect was found within the JVS and UWT group in any of the measures. No other significant interaction effects between stress exposure and treatment were found.

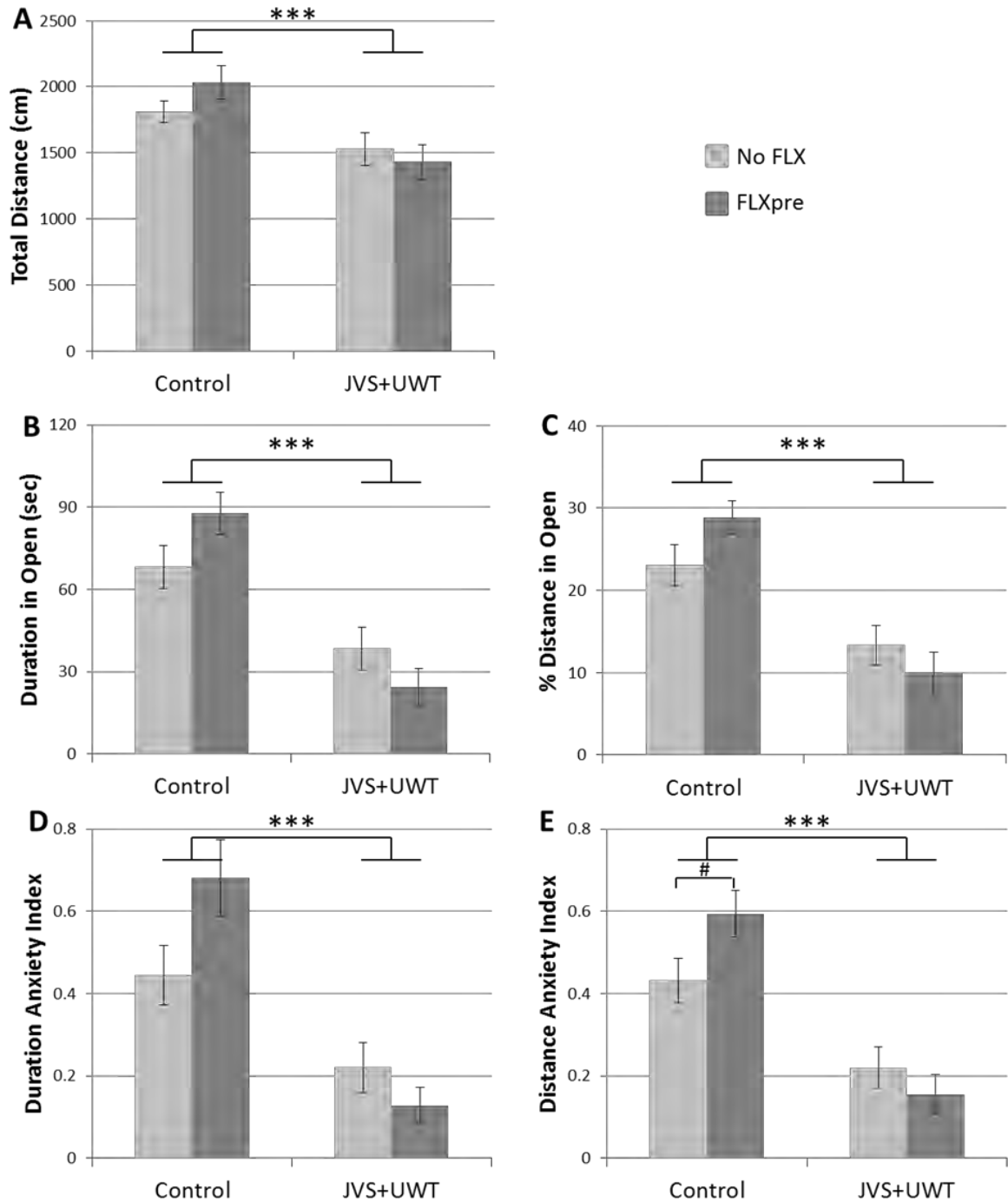


Fig 4-1 Effects of stress exposure (JVS & UWT) and fluoxetine treatment prior to UWT on anxiety-like behavior in the elevated plus maze. Data expressed in means \pm SEM. Rats that were exposed to JVS and UWT, whether or not they were treated with FLX following UWT, covered less total distance in the maze (A), spent less time (B) and covered less distance (C) in the open arms, and exhibited higher levels of anxiety in both duration and distance indices (D & E respectively; indices are measured as open / closed ratio, thus lower ratios indicate higher anxiety levels), compared to control rats. FLX treatment significantly lowered anxiety-like behavior only within the control group in the distance anxiety index (E). No other effects were found. *- Main effect for stress exposure, $p < .05$, **- $p < .01$. #- compared to control, $p < .05$. [N: control-13, control+FLXpre-12, JVS+UWT-12, JVS+UWT+FLXpre-13].

Clearly there was no substantial beneficial effect of the FLX treatment, even when given since juvenility and until UWT, on the rats' anxiety-like behavior as measured by the EPM test. The results here are quite similar to what we found when the FLX treatment was given during adulthood, following the UWT.

Behavioral effects of Fluoxetine on long-term effects of juvenile stress alone.

Our previous experiments showed no beneficial effect for FLX at either time point, on the anxiety-like behavior of rats that were exposed to JVS and UWT in adulthood. As shown in the behavioral results of experiment 1 above, all rats that were exposed to JVS showed anxiety-like behavior. There was actually no behavioral difference between rats that were also exposed to UWT later in adulthood and the rats that were not. Thus it is possible that the JVS alone might be a sufficient model for PTSD that was caused during childhood. Therefore we decided to test whether FLX might benefit with rats that were exposed to JVS alone. In this experiment rats were exposed to JVS alone and were treated with FLX for 4-5 weeks. Some were treated with FLX following JVS and until the parallel time point of UWT, and others were treated in adulthood – since the parallel time point of UWT and until EPM test 4 weeks later.

Methods

Animals

Male Sprague Dawley rats (22 days old, 30-50 g) were used for the experiment. Animals were housed in groups of ~3, at $22 \pm 2^{\circ}\text{C}$ under 12-h light/dark cycles with water and food ad libitum. The experiment was approved by the University of Haifa Ethics and Animal Care Committees.

Experimental groups

Following acclimation all rats were randomly assigned to one of the following experimental conditions:

JVS+FLXpre - Rats were exposed to JVS and treated with FLX for approx. 5 weeks, following the JVS and until adulthood (the timing of UWT in the previous experiments).

JVS+FLXpost - Rats were exposed to JVS and treated with FLX in adulthood for 4 weeks since the timing of UWT in the previous experiments and until the EPM test.

JVS - Rats were exposed to JVS alone.

Control – Rats were not exposed to any stress procedure.

Experimental design

The experimental design was similar to the previous experiments, without the UWT procedure and with FLX treatment at different periods. Following JVS, rats in JVS+FLXpre group were treated chronically with fluoxetine via drinking water for approximately 5 weeks until the UWT time point as detailed above in experiment 4. Rats from the JVS+FLXpost group were treated chronically with fluoxetine according to the same protocol for 4 weeks since the UWT time point and until EPM test. All other rats were receiving regular drinking water.

Statistical analysis.

In order to test also for interaction effects, we analyzed data from this experiment together with data from previous experiments (Rats that were not exposed to any stress, but were treated with FLX following the JVS and until adulthood, or during adulthood; Control+FLXpre and Control+FLXpost, respectively). Data was analyzed using Two-way ANOVA, with LSD Post-Hoc comparisons.

Results

As depicted in figure 5-1, Two-way ANOVA of JVS exposure X FLX treatment revealed a significant main effect for JVS exposure on total distance covered [$F(1,86)=5.49$, $p<0.05$], duration of stay in the Open arms [$F(1,86)=10.9$, $p=0.001$], percent of distance covered in the open arms [$F(1,86)=14.53$, $p<0.001$], as well as on both duration and distance anxiety indices [$F(1,86)=13.72$, $p<0.001$; $F(1,86)=18.63$, $p<0.001$; respectively]. Rats that were exposed to JVS, whether or not they were treated with FLX at any time point, covered significantly less distance in the total maze, spent significantly less time and covered less distance in the open arms, and exhibited significantly higher levels of anxiety in both duration and distance indices (lower ratios indicate higher anxiety levels), compared to control rats.

There was also found a significant main effect for FLX treatment on duration of stay in the Open arms [$F(2,86)=9.38$, $p<0.001$], percent of distance covered in the open arms [$F(2,86)=8.78$, $p<0.001$], as well as on both duration and distance anxiety indices [$F(2,86)=8.99$, $p<0.001$; $F(2,86)=10.69$, $p<0.001$; respectively]. Post-Hoc comparisons revealed a significant difference

between non-treated rats and rats that were treated with FLX during adulthood ($p < 0.01$). Rats that were treated with FLX during adulthood until the EPM test (FLXpost), whether or not they were exposed to JVS, spent significantly less time and covered less distance in the open arms, and also exhibited significantly higher levels of anxiety in both duration and distance indices, compared to non-treated rats.

There was not found any significant interaction effect between JVS exposure and FLX treatment on any of the measures.

Apparently FLX treatment during adulthood and until the EPM test, whether or not rats were exposed to JVS, increased anxiety-like behavior in this experiment. This might be due to FLX ongoing effect during the test. However, FLX treatment since juvenility, immediately following JVS and until adulthood, 4 weeks before behavioral testing, actually showed a slight reduction in anxiety-like behavior.

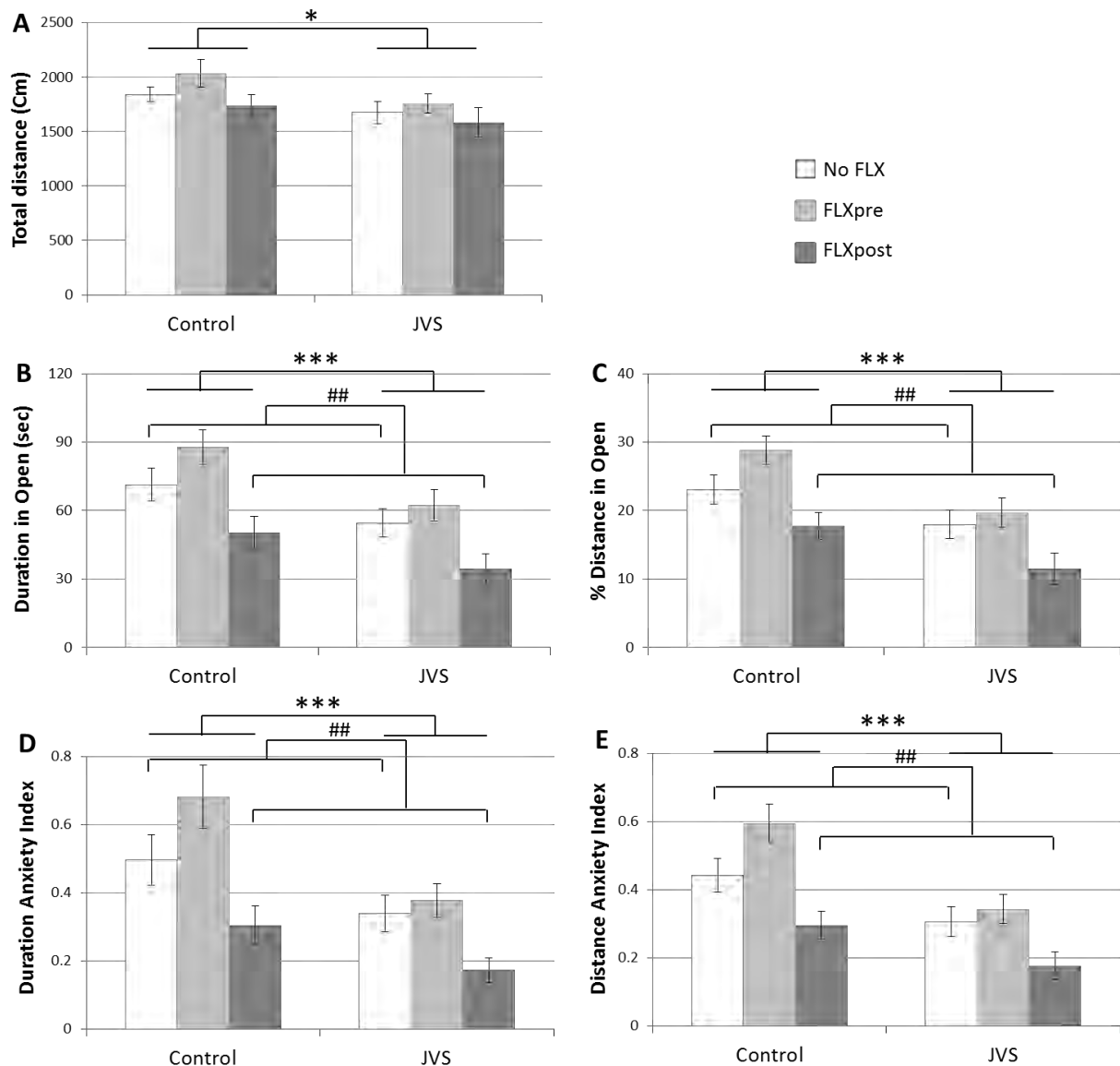


Fig 5-1 Effects of JVS exposure and fluoxetine treatment on anxiety-like behavior in the elevated plus maze. Data expressed in means \pm SEM. Rats that were exposed to JVS, whether or not they were treated with FLX at any time point, covered less total distance in the maze (A), spent less time (B) and covered less distance (C) in the open arms, and exhibited higher levels of anxiety in both duration and distance indices (D & E respectively; indices are measured as open / closed ratio, thus lower ratios indicate higher anxiety levels), compared to control rats. FLX treatment during adulthood (FLXpost) significantly lowered time spent in the open arms (B) and distance covered in the open arms (C), and increased levels of anxiety in both duration and distance indices (D & E respectively), compared to non-treated rats. No interaction effects were found. *- Main effect for JVS exposure, $p < .05$, ***- $p < .001$. ##- Main effect for treatment, compared to non-treated rats, $p < .01$. [N: control-19, control+FLXpre-12, control+FLXpost-12, JVS-18, JVS+FLXpre-19, JVS+FLXpost-12].

A behavioral profiling approach - Summary of Fluoxetine treatment experiments

Our results detailed above indicate no substantial beneficial effect for FLX treatment in the stressed rats in our model. However, there seem to be a slight trend of reduction in anxiety-like behavior in EPM in rats that were exposed to JVS alone and treated with FLX immediately following the stress and until adulthood, 4 weeks before the behavioral test.

Behavioral Profiling Approach

Since not all individuals develop PTSD following a trauma, we believe that comparing only groups' means is not the adequate type of analysis. Thus we employed a behavioral profiling approach (adapted from Cohen et al., 2004; Matar et al., 2013; Horowitz et al., 2014), which analyzes individual differences compared to the norm. In order to create a behavioral classification, we referred to the performance of the control group from all FLX experiments (n=31) as the behavior of the normal population. We first determined the distribution of measures in the control group and calculated the lower 20th percentages (or upper depending on the measure). This approach was applied on 7 different parameters of distance and duration in the EPM, which represent anxiety-like behaviors. The measures of each animal were compared to the distribution curve of the control group. In order to be classified as affected, an animal must exhibit values that are under/above the 20th percentages in at least 4 of the 7 measures. Figure 1 shows the representative performances of an "affected" animal.

Statistical analysis.

Data collected from all experiments was analysed using Chi-squared test for goodness of fit, testing the proportion of affected animals in each group. The distribution between affected and non-affected animals in each group was compared to the expected distribution in the control group (20:80, respectively).

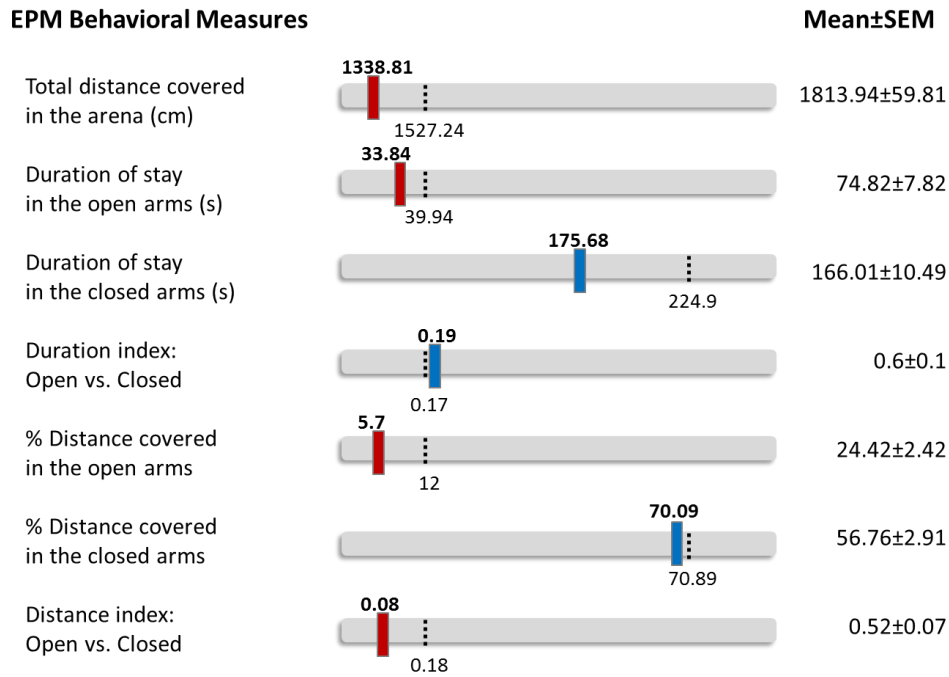


Fig 1 A representative figure of the performances of an "affected" animal

Results of Behavioral Profiling

As depicted in figure 2, Chi-squared test for goodness of fit to a distribution ratio of 20:80 for affected/non-affected animals, revealed no significant difference for the distributions of the different control groups. The test did reveal a significant difference for the distributions of JVS [$\chi^2_{(1)} = 4.65$, $p < 0.05$], JVS+FLXpost [$\chi^2_{(1)} = 11.02$, $p < 0.001$] and the different JVS+UWT groups [untreated JVS+UWT: $\chi^2_{(1)} = 10.01$, $p < 0.01$; JVS+UWT+FLXpre: $\chi^2_{(1)} = 19.69$, $p < 0.001$; JVS+UWT+FLXpost: $\chi^2_{(1)} = 16.33$, $p < 0.001$]. There was also no significant difference for the distribution of JVS+FLXpre. Rats that were exposed to JVS, whether or not they were also exposed to UWT during adulthood, exhibited significantly higher rates of affected animals. Only when rats were exposed to JVS alone and treated with FLX since juvenility, their proportion of affected animals was similar to controls.

Within animals that were exposed to JVS only, Chi-squared test for goodness of fit to the distribution ratio of untreated JVS rats (35:65) for affected/non-affected animals, revealed a significant difference only for the distribution of rats that were treated with FLX since juvenility [JVS+FLXpre: $\chi^2_{(1)} = 5$, $p < 0.05$].

Within animals that were exposed to JVS and UWT, Chi-squared test for goodness of fit to the distribution ratio of untreated JVS+UWT rats (46:54) for affected/non-affected animals, revealed no significant difference for the distribution of rats treated with FLX, whether the treatment was since juvenility or only in adulthood.

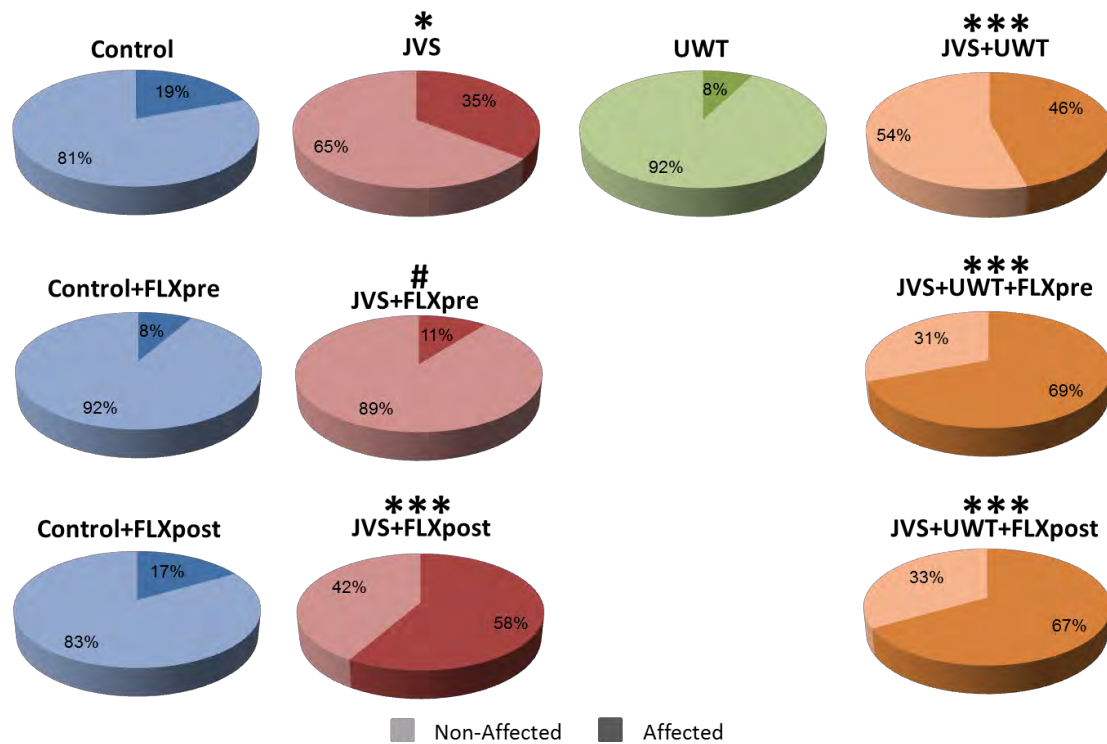


Fig 2: The effect of juvenile stress and underwater trauma on the distribution of affected and non-affected animals according to behavioral profiling approach. Rats that were exposed to JVS, whether or not they were also exposed to UWT during adulthood, exhibited a significantly larger proportion of affected animals. Within the stressed rats that were treated with FLX, only rats that were exposed to JVS alone and were treated since juvenility exhibited a proportion similar to the controls. *- compared to control's distribution, $p < .05$, ***- $p < .001$. #- compared to JVS alone group's distribution, $p < .05$. [N: control-31, control+FLXpre-12, control+FLXpost-12, JVS-31, JVS+FLXpre-19, JVS+FLXpost-12, UWT-13, JVS+UWT-24, JVS+UWT+FLXpre-13, JVS+UWT+FLXpost].

These results indicate that FLX treatment to rats exposed to JVS and UWT, at either time point, did not lower the affected proportion of affected animals. However, FLX treatment during juvenility, given to rats that were only exposed to JVS, did lower significantly the proportion of affected animals to a similar proportion as in the control group. These results suggest that juvenility is not only a sensitive period for vulnerability but it may also serve as a window for recovery.

Task 7a: Efficacy of intervention – Non pharmacological interventions –

In addition to examining the efficacy of potential pharmacological interventions we have initiated the examination of the efficacy of non-pharmacological interventions. Because our model involves two steps, i.e. the exposure to juvenile stress that induces vulnerability and the exposure to the adulthood trauma, we attempt to examine the impact of such interventions on vulnerability (i.e. immediately after the juvenile stress) and on the pathology (only after the exposure also to the trauma in adulthood).

Pre-pubertal ('juvenile') stress-induced susceptibility to PTSD, which is associated with selective alterations in GABAAR alpha1 subunit in the dentate and amygdala, is rescued by juvenile but not adulthood exposure to 'Enriched Environment'

Introduction

Animal studies demonstrate that enriched environment (EE) treatment counteracts cognitive deficits induced by stress early in life (Guilarte, Toscano, McGlothan & Weaver, 2003; Helleman, Benge & Olmstead, 2004) and rescues abnormal behaviors, such as anxiety like behaviors induced by stress in adulthood (Chapillon et al., 1999; Engellenner et al., 1982; Hansen, 2000). Recent data from our lab suggests that an exposure to stress in juvenility exacerbates the behavioral effects of an exposure to stress in adulthood (Horovitz et al, 2012; 2014) which was associated with selective alterations in GABAAR alpha1 subunit in the ventral and dorsal dentate gyrus and amygdala.

Thus, the aim of the current study was to examine the ability of 'Enriched Environment' in juvenility or in adulthood to prevent these effects of the exposure to stress in adulthood on the background of previous exposure to stress in juvenility.

Methods

a. Juvenile stress: 3 consecutive days of exposure to acute stressors: 27PND – forced swim stress (10min.), 28PND – elevated platform (30min. X 3, ITI60min. in home cage), and 29PND – confinement (120min.).

b. Enriched Environment: Animals were transferred to the EE cages at 30PND or 60±PND. Sawdust bedding was replaced once a week and toys were replaced twice a week.

c. Under water trauma (+ odor): After 2 min. habituation to the room, rats were exposed to a vanilla odor inside the cage for 30 sec. and then immediately to the UWT stress (5 s swim in a water tank (50x60x60 cm) and then rats are held under water for 45 s, using a special metal net.)

d. Odor re exposure: After 2 min. habituation to the room, rats were exposed to a vanilla odor inside the cage for 30 sec.

e. Behavioral assessments: Elevated plus maze test: 8 min. testing under full light.

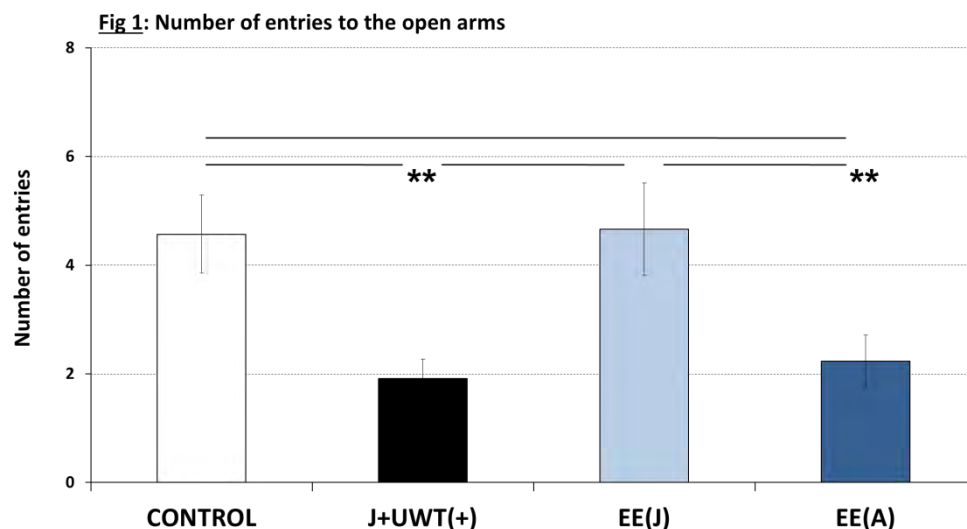
f. Tissue collection: Brains were collected and snap frozen on dry ice.

- **Expression changes GABAAR alpha1 subunit in the ventral and dorsal dentate gyrus and amygdala were assessed by western blot, as described in (Ritov et al., 2014)**

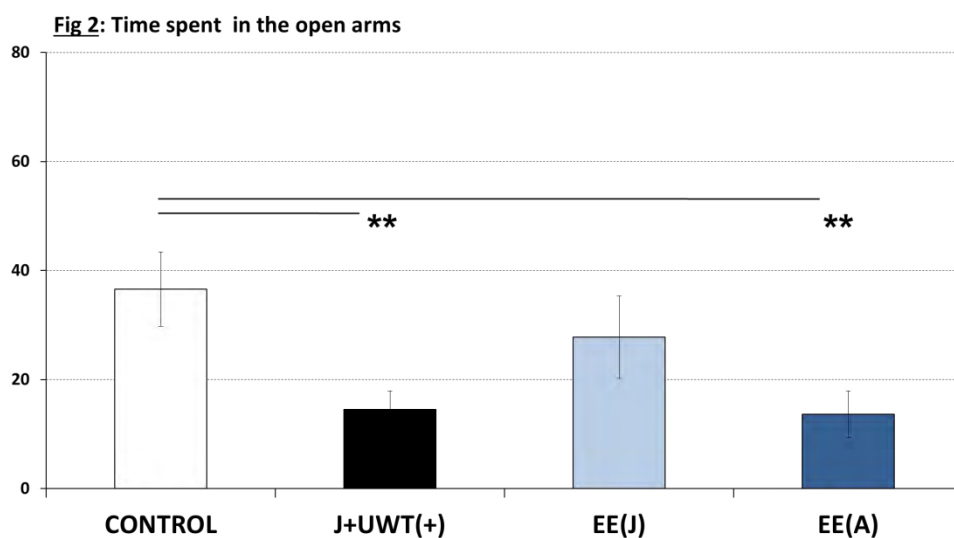
Procedures and Ages							
Groups	Juvenile Stress ^a	Enriched Environment ^b	UWT (+ odor) ^c	Enriched Environment	Odor Re exposure ^d	Behavioral Assessments ^e	Tissue collection ^f
<i>PNDs'</i>	27-29	30-60	60	60-90	91		92
Control (n=14)	-	-	+	-	+	+	+
			(odor only)				
EE (J) (n=12)	+	+	+	-			
EE (A) (n=13)	+	-	+	+			
JS+UWT(+) (n=12)	+	-	+	-			

Results

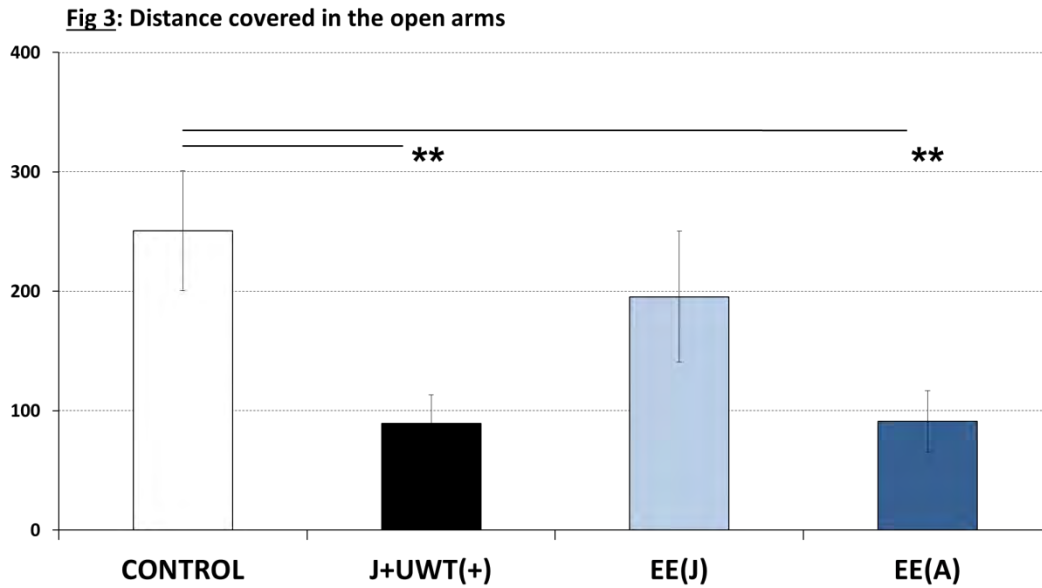
Anxiety like behaviors in the Elevated Plus Maze:



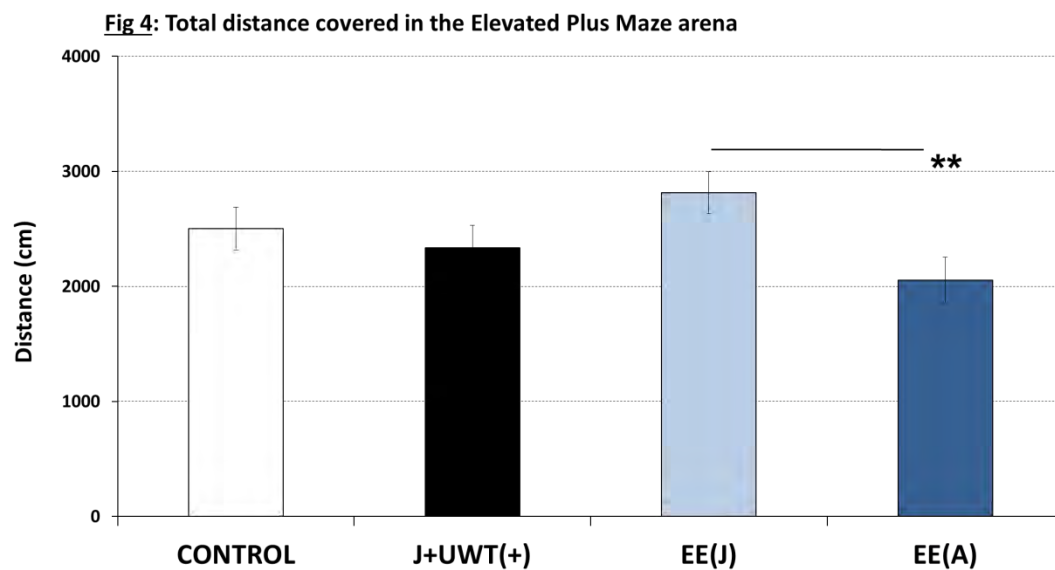
1: One way ANOVA [$F_{(3,47)} = 5.36$, $p < 0.01$] revealed a significant main effect for the exposure. Further Post hoc comparisons indicated that both J+UWT(+) and EE(A) rats' enter less times to the open arms of the EPM compared to Control and EE(J) rats' . (**= $p < 0.01$)



2: One way ANOVA [$F_{(3,47)} = 3.75$, $p < 0.05$] revealed a significant main effect for the exposure. Further Post hoc comparisons indicated that both J+UWT(+) and EE(A) rats' spent less time in the open arms of the EPM compared to Control. (**= $p < 0.01$)

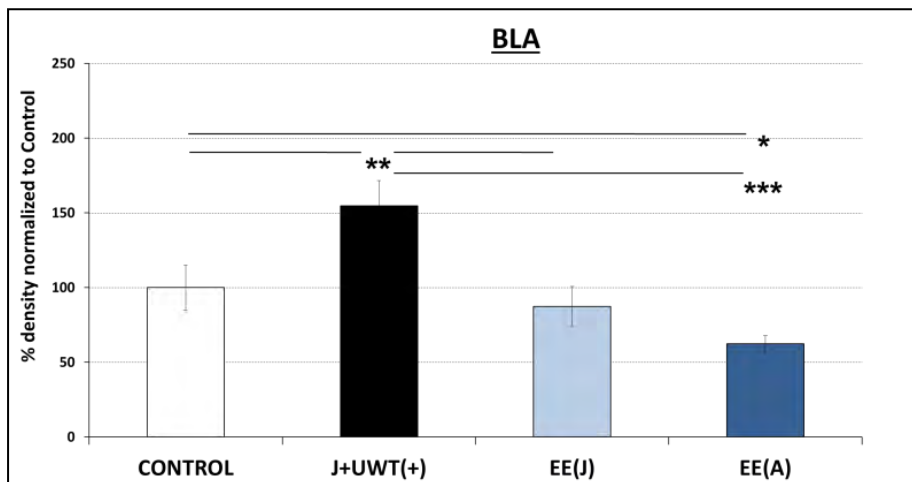
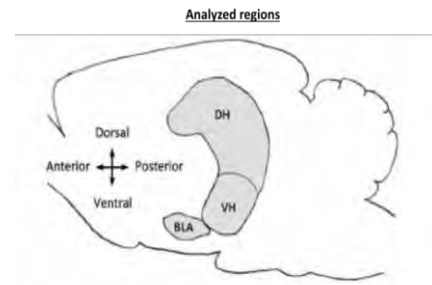


3: One way ANOVA [$F_{(3,47)} = 3.80$, $p < 0.05$] revealed a significant main effect for the exposure. Further Post hoc comparisons indicated that both J+UWT(+) and EE(A) rats' covered less distance in the open arms of the EPM compared to Control. (**= $p < 0.01$)

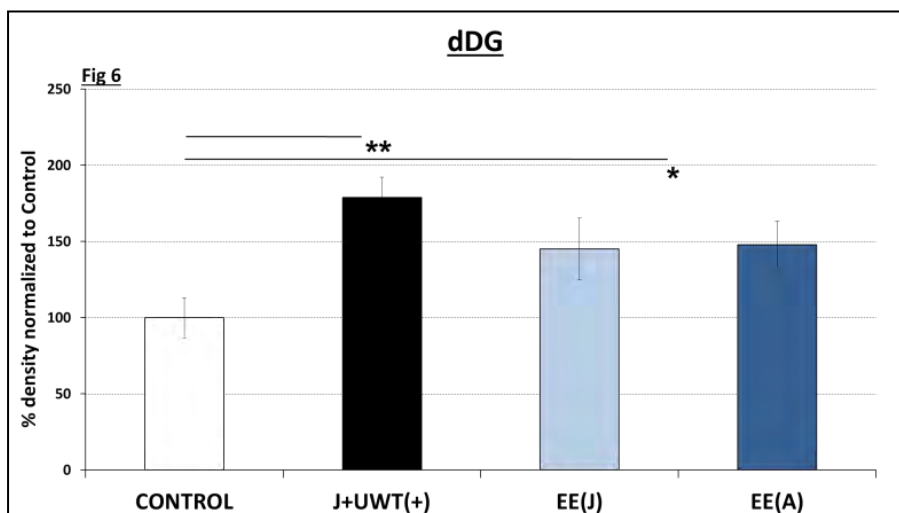


4: One way ANOVA [$F_{(3,47)} = 2.70$, $p < 0.05$] revealed a significant main effect for the exposure. Further Post hoc comparisons indicated that EE(A) rats' covered less distance in the EPM arena compared to EE(J) rats' . (**= $p < 0.01$)

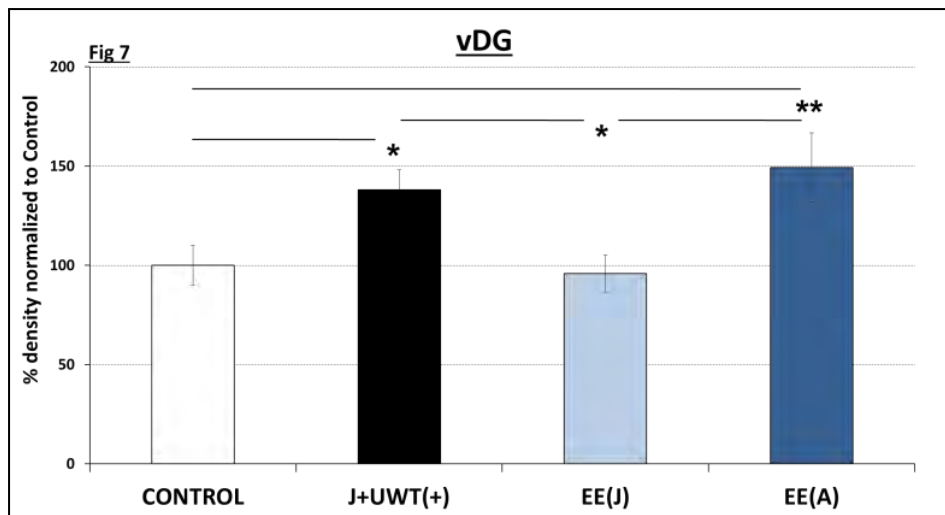
GABAAR alpha 1 expression in Amygdala and dorsal VS ventral Dentate Gyrus



5: One way ANOVA [$F_{(3,47)} = 8.27$, $p < 0.001$] revealed a significant main effect for the exposure. Further Post hoc comparisons revealed increased BLA GABAAR alpha 1 expression in J+UWT(+) rats compared to Control, EE (J) and EE (A) rats. In addition, EE (A) rats exhibit lower expression of BLA GABAAR alpha 1 expression compared to Control. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).



6: One way ANOVA [$F_{(3,47)} = 4.44$, $p < 0.01$] revealed a significant main effect for the exposure. Further Post hoc comparisons revealed increased dDG GABAAR alpha 1 expression in both J+UWT(+), EE (J) and EE (A) rats compared to Control. In addition, EE (A) rats exhibit lower expression of BLA GABAAR alpha 1 expression compared to Control. (* = $p < 0.05$, ** = $p < 0.01$).



7: One way ANOVA [$F(3,47) = 4.79$, $p < 0.01$] revealed a significant main effect for the exposure. Further Post hoc comparisons revealed increased vDG GABAAR alpha 1 expression in both J+UWT(+) and EE (A) rats compared to EE (J) and Control rats'. (* = $p < 0.05$, ** = $p < 0.01$)

Summary

While an exposure to EE in adulthood was ineffective in preventing the behavioral effects of an exposure to the combination of 'Juvenile + adulthood stress', EE in juvenility could prevent these effects.

In addition, while GABAAR alpha1 subunit levels in dorsal hippocampus remained high, an exposure to EE in juvenility could restore the expression levels back to control levels in both BLA and ventral DG. In contrast, an exposure to EE in adulthood results in lower expression levels of GABAAR alpha1 in the BLA compared to control and was ineffective in restoring the expression levels in ventral DG back to control levels.

Conclusions

- These results suggest that "juvenility" is a sensitive period not only with regard to stress vulnerability but also for stress resilience and therefore may serve as a critical point for intervention.
- In addition, these results highlight GABAAR alpha1 subunit specifically in the BLA and in ventral DG as potential neurobiological correlates of the protective abilities of an exposure to enriched environment in juvenility.

Key research accomplishments

Towards the end of the fourth year of the project, the following can already be indicated as research accomplishments:

- **The Under-water Trauma model:** The UWT model, which is an ethological model of a brief but intense traumatic event (Richter-Levin, 1998) was further developed here in a way that is of particular relevance to combat soldiers. It was found to have an impact by itself, and also to be a convenient platform for examining the added impact of relevant risk factors.

This model has already been adopted in other laboratories, e.g:

Moore NL, Gauchan S, Genovese RF. (2012) Differential severity of anxiogenic effects resulting from a brief swim or underwater trauma in adolescent male rats. *Pharmacol Biochem Behav.* ;102(2):264-8.

Adamec R, Toth M, Haller J, Halasz J, Blundell J. (2012) A comparison of activation patterns of cells in selected prefrontal cortical and amygdala areas of rats which are more or less anxious in response to predator exposure or submersion stress. *Physiol Behav.* 105(3):628-38.

Adamec R, Toth M, Haller J, Halasz J, Blundell J. (2012) Activation patterns of cells in selected brain stem nuclei of more and less stress responsive rats in two animal models of PTSD - predator exposure and submersion stress. *Neuropharmacology.* 62(2):725-36.

Moore NL, Gauchan S, Genovese RF. (2014) Adolescent traumatic stress experience results in less robust conditioned fear and post-extinction fear cue responses in adult rats. *Pharmacol Biochem Behav.* 120:17-24.

- **The Pre-pubertal (Juvenile) stress exposure as a risk factor in PTSD:** We have brought strong evidence for the importance of considering risk factors in PTSD. While intuitively it is tempting to think of the exposure to the trauma as the cause of PTSD the fact that the large majority of individuals exposed to trauma do not develop PTSD indicates that this is not the case. Only when additional risk factors are present can a trauma lead to PTSD. This means that understanding the neural mechanisms of these risk factors is critical for developing an effective prevention treatment. We have successfully established a model of one very relevant risk factor, a model which enables us now to study the neural mechanisms involved.

This model has also already been adopted by other laboratories, e.g:

Maggio N, Segal M. (2011) Persistent changes in ability to express long-term potentiation/depression in the rat hippocampus after juvenile/adult stress. *Biol Psychiatry.* 69(8):748-53.

Brydges NM, Jin R, Seckl J, Holmes MC, Drake AJ, Hall J. (2014) Juvenile stress enhances anxiety and alters corticosteroid receptor expression in adulthood. *Brain Behav.* 4(1):4-13.

Brydges NM, Wood ER, Holmes MC, Hall J. (2014) Prepubertal stress and hippocampal function: sex-specific effects. *Hippocampus.* 24(6):684-92.

Fuentes S, Carrasco J, Armario A, Nadal R. (2014) Behavioral and neuroendocrine consequences of juvenile stress combined with adult immobilization in male rats. *Horm Behav.* 66(3):475-86.

Tzanoulinou S, Riccio O, de Boer MW, Sandi C. (2014) Peripubertal stress-induced behavioral changes are associated with altered expression of genes involved in excitation and inhibition in the amygdala. *Transl Psychiatry.* 4:e410.

- **The introduction of a trauma-reminder cue into the model:** The maladaptive response of PTSD patients to reminder cues of the traumatic events is a hallmark of the disorder. We have incorporated this important component into our model. This has enabled us -
 - a) To improve the sensitivity of the model as a drug testing platform.
 - b) To better understand variables which contribute to the effectiveness of reminder cues (in order to guide treatment).
 - c) To use the model to elucidate the neural mechanisms associated with abnormal responses to reminder cues.
- **The focus on long-term effects of trauma exposure:** The finding that PTSD symptoms in this model last for over four weeks establishes it as a relevant model but also enables utilizing this model for long-term drug treatment at different time points following the exposure to the traumatic event.
- **The Behavioral profiling approach – Translational diagnosis:** Diagnosis of psychiatric disorders in humans is based on comparing individuals to a normal population. In contrast, animal models tend to analyze averaged group effects instead, thus compromising their translational power. This discrepancy is particularly relevant to posttraumatic stress disorder (PTSD), because only a subset of individuals exposed to a traumatic experience eventually develop the disorder. We have developed a novel approach – *Behavioral Profiling* – with which we are able to identify exposed-affected from exposed non-affected individuals, in a way similar to the human practice. Beyond achieving improved validity of the model, the identification of affected versus exposed, non-affected individuals should enable more accurate association with pathology-related- but also with resilience-related neural mechanisms.
- **A rat model of high relevance to PTSD was confirmed:** Generally, animal models are required to demonstrate mainly three types of validation criteria: Construct validation - which in a simple way means that it is possible to set up in the animal the mechanisms of the disease. However, this is not possible in the case of psychopathologies like PTSD because the mechanism of these disorders is not known. Pharmacological validation – This requires that drugs that are known to be effective in treating the disease in humans will reduce symptoms in the animal model. This type of validation is often presented but in fact can be challenged because of the lack of gold standard medication in PTSD. The efficacy of currently available drugs is very partial, with a high percent of partial responders and non-responders. It would thus be questionable to conclude that no effect of a

currently available drug indicates inadequacy of a model, or that the success of a drug in current models is a validation of the model.

Finally, probably the weakest form of validation is face validity, which in simple terms requires that the symptoms the animal presents hold similarity to the human pathology.

Because of the difficulty in presenting reliable validation of any model we have set out here to ensure a level of face validity to our model that will be stronger than other models before. This was achieved by combining an ethologically-relevant trauma (the UWT), combining it with a validated risk factor (the Juvenile stress), and with measuring the response to a trauma-reminder cue (odor or water context), and most significantly, analyzing the results based on the translational Behavioral Profiling approach, which differentiates between exposed-affected and exposed-unaffected individuals.

Combined together, this is currently the most relevant animal model of PTSD, ready to be exploited.

Reportable outcomes

Published Manuscripts (in which the support of the DOD is indicated):

Sood R, Ritov G, **Richter-Levin G**, Barki-Harrington L. (2012) Selective increase in the association of the β 2 adrenergic receptor, β Arrestin-1 and p53 with Mdm2 in the ventral hippocampus one month after underwater trauma. Behavioral Brain Research. 240:26-8.

Schmidt MV, Abraham WC, Maroun M, Stork O, **Richter-Levin G**. (2013) Stress-induced metaplasticity: From synapses to behavior. *Neuroscience*. 250:112-20.

Zitman FM, **Richter-Levin G**. (2013) Age and sex-dependent differences in activity, lasticity and response to stress in the dentate gyrus. *Neuroscience*. 249:21-30.

Ritov G, **Richter-Levin G**. (2014) Water associated zero maze: a novel rat test for long term traumatic re-experiencing. *Frontiers in Behavioral Neuroscience*. 17;8:1.

Horovitz O, Tsoory MM, Yovell Y, **Richter-Levin G**. (2014) A rat model of pre-puberty (Juvenile) stress-induced predisposition to stress-related disorders: Sex similarities and sex differences in effects and symptoms. *World Journal of Biological Psychiatry*. 15(1):36-48.

Ardi Z, Ritov G, Lucas M, **Richter-Levin G**. (2014) The effects of a reminder of underwater trauma on behavior and memory-related mechanisms in the rat dentate gyrus. *International Journal of NeuroPsychoPharmacology*, 17(4):571-80.

Fa M, Xia L, Anunu R, Kehat O, Kriebel M, Volkmer H, **Richter-Levin G**. (2014) Stress modulation of hippocampal activity - spotlight on the dentate gyrus. *Neurobiology of Learning and Memory*. 112:53-60.

Ritov G, Ardi Z, **Richter-Levin G**. (2014) Differential activation of amygdala, dorsal and ventral hippocampus following an exposure to a reminder of underwater trauma. *Front Behav Neurosci*. 8:18.

Sood R, Ritov G, Boltyansky B, Spector-Chotiner A, **Richter-Levin G**, Barki-Harrington L. (2014) Underwater trauma causes a long-term specific increase in the expression of cyclooxygenase-2 in the ventral CA₁ of the hippocampus. *Psychoneuroendocrinology*. 49:62-8.

Abstracts in meetings during the fourth year:

Albrecht A., Ardi Z., Richter-Levin G. (2014) Long-term alterations in neuropeptide gene expression in sublayers of the dorsal dentate gyrus in a rat model of Posttraumatic stress disorder. The 23rd Annual Meeting of Israel Society for Neuroscience, Israel.

Albrecht A., Ivens S., Calışkan G. , Heinemann U., Richter-Levin G., Stork O. (2014) Shifts in excitatory/inhibitory balance by juvenile stress: a role for neuron-astrocyte interaction in the dentate gyrus. The 23rd Annual Meeting of Israel Society for Neuroscience, Israel.

Ariel L., Edut S., Inbar S., Richter-Levin G (2014) Fluoxetine reduces the prevalence of affected individuals following pre-puberty but not adulthood trauma in an animal model of PTSD. The 23rd Annual Meeting of Israel Society for Neuroscience, Israel.

Ritov G., Boltyansky B., Richter-Levin G. (2014) Administration of Methylphenidate before exposure to trauma attenuates anxious reactions to a reminder one month later. The 23rd Annual Meeting of Israel Society for Neuroscience, Israel.

Ritov G., Boltyansky B., Richter-Levin G. (2014) Combining behavioral profiling and network excitation analysis reveals functionally distinct patterns of pathology in a rat model of traumatic re-experiencing. The 23rd Annual Meeting of Israel Society for Neuroscience, Israel.

Richter-Levin G. (2014) The way stress re-maps memory systems in our brain. The Third International Congress on Alcoholism and Stress. Volterra, Italy.

Richter-Levin G. (2014) Effects of stress on cognitive and neuronal function in animal models. The 22nd Alzheimer's Disease Conference. Tel Aviv University, Israel.

Richter-Levin G (2014) Towards an effective animal model of PTSD. The 2nd Brain Disease Research Center (BDRC) meeting. The Hebrew University, Jerusalem, Israel.

Richter-Levin G., Ardi Z., Albrecht A., Richter-Levin A. (2014) Pre-pubertal ('juvenile') stress-induced susceptibility to PTSD, which is associated with selective alterations in GABAAR alpha1 subunit in the dentate and amygdala, is rescued by juvenile but not adulthood exposure to 'Enriched Environment'. The Annual Society for Neuroscience Meeting, Washington (DC), USA.

Ariel L., Edut S., Inbar S., Richter-Levin G. (2015) Long-term alterations in GABAergic and glucocorticoid receptors' gene expression in a rat model of Posttraumatic stress disorder. The 19th Israel Society For Biological Psychiatry Meeting, Israel.

Ritov G., Boltyansky B., Richter-Levin G. (2015) Methylphenidate administration before trauma exposure attenuates anxious reactions to a reminder one month later. The 19th Israel Society For Biological Psychiatry Meeting, Israel.

Albrecht, A., Ben-Yishay, E., Richter-Levin, G. (2015) A functional role for the dentate gyrus in a rat model of Posttraumatic stress disorder. The 19th Israel Society For Biological Psychiatry Meeting, Israel.

Conclusions

This report is of the Fourth year project report. The project is set to examine the impact of two risk factors (Childhood stress and sleep restriction) for the development of PTSD, to establish an effective platform for drug testing and to identify potential novel targets for drug development in PTSD.

We have explored the potential contribution of sleep restriction to developing PTSD. Contrary to the hypothesis our results do not support such a role for sleep restriction.

We have made excellent progress in establishing childhood stress as a risk factor, and in identifying neural mechanisms associated with this risk factor, that may serve as novel potential targets for developing new drugs. We continue with this line of research into the 5th year.

We would like to state that we strongly believe that the PTSD model we have developed is superior to all animal models of PTSD currently in used for the following reasons:

1. This is the only model that takes into consideration the fact that not all individuals exposed to a traumatic event will eventually develop PTSD.
2. This model takes into consideration individual differences, thus enabling a more accurate association of neuronal alterations with behavioral outcomes.
3. This model looks into long-term effects (four weeks after the exposure to the trauma), thus increasing its relevance to PTSD-related factors.
4. This model includes the impact of reminder cues, much like the human case.

We believe it is ready for a wider utilization towards elucidating neural mechanisms associated with PTSD and with stress resilience. We will be happy to share that knowledge and to help set and train any group interested in this model – for the benefit of promoting the development of effective treatment to PTSD.

We have established an effective platform for drug testing. We have started to conduct drug testing, to further validate the procedure and this goal will continue into the 5th year.

Several papers were published and additional manuscripts are currently in preparation.

It can be stated that progress is good and that the main aims of the project will be achieved as planned.

References

- Adamec R, Hebert M, Blundell J, Mervis RF. (2012) Dendritic morphology of amygdala and hippocampal neurons in more and less predator stress responsive rats and more and less spontaneously anxious handled controls. *Behav Brain Res.* 226(1):133-46.
- Ardi, Z, Ritov G, Lucas M, Richter-Levin G (2014) The effects of a reminder of underwater trauma on behavior and memory-related mechanisms in the rat dentate gyrus. *Int J Neuropsychopharmacol*, 17: 571-580.
- Berridge, K. C. & Kringelbach, M. L. (2013). Neuroscience of affect: brain mechanisms of pleasure and displeasure. *Curr Opin Neurobiol*, 23(3), 294-303.
- Chapillon P, Manneché C, Belzung C, Caston J. (1999) Rearing environmental enrichment in two inbred strains of mice: 1. Effects on emotional reactivity. *Behav Genet.* 29(1):41-6.
- Cohen, H., Matar, M. A. and Zohar, J. (2014). Maintaining the clinical relevance of animal models in translational studies of post-traumatic stress disorder. *ILAR J.* 55(2): 233-245.
- Cohen H, Zohar J, Matar MA, Zeev K, Loewenthal U, Richter-Levin G. (2004) Setting apart the affected: the use of behavioral criteria in animal models of post traumatic stress disorder. *Neuropsychopharmacology.* 29(11):1962-70.
- Conner, K. R., Bossarte, R. M., He, H., Arora, J., Lu, N., Tu, X. M. and Katz, I. R. (2014). Posttraumatic stress disorder and suicide in 5.9 million individuals receiving care in the veterans health administration health system. *J Affect Disord*, 166, 1-5.
- Coulter DA, Carlson GC. (2007) Functional regulation of the dentate gyrus by GABA-mediated inhibition. *Prog Brain Res.* 163:235-43.
- Engellenner WJ, Goodlett CR, Burright RG, Donovan PJ. (1982) Environmental enrichment and restriction: effects on reactivity, exploration and maze learning in mice with septal lesions. *Physiol Behav.* 29(5):885-93.
- File SE. (1993) The interplay of learning and anxiety in the elevated plus-maze. *Behav Brain Res.* 58(1-2):199-202.
- Guilarte TR, Toscano CD, McGlothan JL, Weaver SA. (2003) Environmental enrichment reverses cognitive and molecular deficits induced by developmental lead exposure. *Ann Neurol.* 53(1):50-6.
- Hansen LT, Berthelsen H. (2000) The effect of environmental enrichment on the behaviour of caged rabbits (*Oryctolagus cuniculus*). *Appl Anim Behav Sci.* 68(2):163-178.

- Heim C, Nemeroff CB (2001) The role of childhood trauma in the neurobiology of mood and anxiety disorders: preclinical and clinical studies. *Biol Psychiatry* 49: 1023-1039.
- Hellemans KG, Benge LC, Olmstead MC. (2004) Adolescent enrichment partially reverses the social isolation syndrome. *Brain Res Dev Brain Res.* 150(2):103-15.
- Hoffman SW1, Virmani S, Simkins RM, Stein DG. (2003) The delayed administration of dehydroepiandrosterone sulfate improves recovery of function after traumatic brain injury in rats. *J Neurotrauma.* 20(9):859-70.
- Horovitz O, Tsoory MM, Hall J, Jacobson-Pick S, Richter-Levin G (2012) Post-weaning to pre-pubertal ('juvenile') stress: a model of induced predisposition to stress-related disorders. *Neuroendocrinology* 95 : 56–64.
- Horovitz O, Tsoory MM, Yovell Y, Richter-Levin G. (2014) A rat model of pre-puberty (juvenile) stress-induced predisposition to stress-related disorders: sex similarities and sex differences in effects and symptoms. *World J Biol Psychiatry.* 15(1):36-48.
- Houser CR (2007) Interneurons of the dentate gyrus: an overview of cell types, terminal fields and neurochemical identity. *Prog Brain Res,* 163: 217-32.
- Jacob MH1, Janner Dda R, Jahn MP, Kucharski LC, Belló-Klein A, Ribeiro MF. (2009) DHEA effects on myocardial Akt signaling modulation and oxidative stress changes in aged rats. *Steroids.* 74(13-14):1045-50.
- Lanius, R. A., Brand, B., Vermetten, E., Frewen, P. A. and Spiegel, D. (2012). The dissociative subtype of posttraumatic stress disorder: rationale, clinical and neurobiological evidence, and implications. *Depress. Anxiety,* 29, 701–708.
- Lanius, R. A., Frewen, P. A., Girotti, M., Neufeld, R. W., Stevens, T. K., & Densmore, M. (2007). Neural correlates of trauma script-imagery in posttraumatic stress disorder with and without comorbid major depression: a functional MRI investigation. *Psychiatry Res,* 155(1), 45-56.
- Lanius, R., Vermetten, E., Loewenstein, R. J., Brand, B., Schmahl, C., Bremner, J. D., et al. (2010). A dissociative subtype of PTSD: Clinical and neurobiological evidence. *The American Journal of Psychiatry,* 167(6), 640-647.
- Maggio N, Segal M. (2011) Persistent changes in ability to express long-term potentiation/depression in the rat hippocampus after juvenile/adult stress. *Biol Psychiatry.* 69(8):748-53.
- Matar, M. A., Zohar, J. and Cohen, H. (2013). Translationally relevant modeling of PTSD in rodents. *Cell Tissue Res.* 354(1):127-39. doi: 10.1007/s00441-013-1687-6.

- McNamara RK, Able JA, Rider T, Tso P, Jandacek R. (2010) Effect of chronic fluoxetine treatment on male and female rat erythrocyte and prefrontal cortex fatty acid composition. *Prog Neuropsychopharmacol Biol Psychiatry*. 34(7):1317-21.
- Meerlo P, Sgoifo A, Suchecki D. (2008) Restricted and disrupted sleep: effects on autonomic function, neuroendocrine stress systems and stress responsivity. *Sleep Med Rev*. 12(3):197-210
- Nemeroff, C. B., Bremner, J. D., Foa, E. B., Mayberg, H. S., North, C. S., & Stein, M. B. (2006). Posttraumatic stress disorder: a state-of-the-science review. *J Psychiatr Res*, 40(1), 1-21.
- Richter-Levin G. (1998) Acute and long-term behavioral correlates of underwater trauma--potential relevance to stress and post-stress syndromes. *Psychiatry Res*. 79(1):73-83.
- Ritov, G., Ardi, Z. and Richter-Levin, G. (2014). Differential activation of amygdala, dorsal and ventral hippocampus following an exposure to a reminder of underwater trauma. *Front Behav Neurosci*, 8: 18.
- Ritov, G, Richter-Levin G (2014): Water Associated Zero Maze: A novel rat test for long term traumatic re-experiencing. *Front Behav Neurosci*, 8: 1.
- Segal M, Richter-Levin G, Maggio N. (2010) Stress-induced dynamic routing of hippocampal connectivity: a hypothesis. *Hippocampus*. 20(12):1332-8.
- Sood, R., Ritov, G., Boltyansky, B., Spector-Chotiner, A., Richter-Levin, G. and Barki-Harrington, L. (2014). Underwater trauma causes a long-term specific increase in the expression of cyclooxygenase-2 in the ventral CA1 of the hippocampus. *Psychoneuroendocrinology*, 49; 62-68.
- Sood, R., Ritov, G., Richter-Levin, G. and Barki-Harrington, L. (2013). Selective increase in the association of the Beta 2 adrenergic receptor, Beta Arrestin-1 and p53 with Mdm2 in the ventral hippocampus one month after underwater trauma. *Behavioural Brain Research*, 240; 26– 28.
- Wang J, Akirav I, Richter-Levin G (2000) Short-term behavioral and electrophysiological consequences of underwater trauma. *Physiol Behav* 70:327-332.